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Biology of *Frankia* Strains, Actinomycete Symbionts of Actinorhizal Plants

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INTRODUCTION

Actinomycetes that make up the genus *Frankia* are distinguished by their ability to induce N_2-fixing root nodules on certain nonleguminous plants (268). After many years of effort, the isolation of the first *Frankia* strain was reported in 1978 by Torrey and colleagues (62). This report demonstrated that *Frankia* strains constituted a previously undescribed group of soil actinomycetes that are facultatively symbiotic with higher plants. To date, strains have been

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isolated from 20 of 24 actinorhizal plant genera, although in several instances, reinfection has not been achieved (Table 1). As a consequence, the genus *Frankia* is now relatively well defined, some species have been proposed, the growth characteristics and physiology of certain strains have been studied in some detail, and the first steps toward developing the molecular genetics of *Frankia* strains have been taken. The striking genetic and phenotypic diversity of available strains and the considerable number of plant species yet to yield infective and effective isolates make these slow-growing actinomycetes intriguing subjects for studies on the evolution of plant-microorganism symbioses and the physiological and genetic interactions that take place in N_2-fixing symbioses. In addition, *Frankia* strains produce three cell types that provide opportunities for studying prokaryotic development.

Several excellent reviews have dealt with actinorhizal plant ecology and potential uses (30, 72, 73, 76, 77), evolution (171), infection processes (49), root nodule physiology (114, 233, 261), cytology (181), *Frankia* genetics (170, 189, 241), microbiology (4), and general aspects (23, 223); this review will address recent information, in light of previous work, on the microbiology of *Frankia* strains, with special emphasis on their taxonomy, ecology, physiology, and genetics.

Actinorhizal Plants

The plants nodulated by *Frankia* strains are known as actinorhizal plants (268). To date, about 194 species and 24 genera of such plants have been identified (Table 1). Unlike the rhizobium-legume symbiosis, in which the host plants, with a few exceptions, belong to a single large family, actinorhizal plants are distributed among eight families and seven orders of angiosperms. These plants have in common a predilection to grow in marginally fertile soils, and they often serve as pioneer species early in successional plant community development. Representatives can be found in most climatic zones, and they inhabit a variety of ecosystems including arctic tundra (*Dryas* species), coastal dunes (*Casuarina*, *Hippophaë*, *Myrica*, and *Elaeagnus* species), riparian (*Alnus* and *Myrica* species), glacial till (Alnus and *Dryas* species), forest (*Alnus*, *Casuarina*, *Coriaria*, and *Shepherdia* species), chapparal and xeric (*Casuarina*, *Purshia*, *Ceanothus*, *Cercocarpus*, *Comptonia*, and *Cowania* species), and alpine (*Alnus* species). The input of fixed nitrogen by these plants can be considerable, especially in colder temperate areas where indigenous legumes are absent or rare (231). Pollen distributions in marine sediment cores have recorded the past roles played by actinorhizal plants in colonizing deglaciated soils during periods of major climatic change (108). In the postglacial period of the early Holocene (10,000 to 8,000 B.P.), *Alnus* became the dominant plant in North America and Europe, eventually accounting for about 40% of the tree pollen in postglacial Britain and North America (231). In Scandinavia, *Hippophaë* and *Alnus* species preceded the present conifer forest, and *Shepherdia* species are considered to have played a similar role in Canada.

Because they often thrive on marginal soils, actinorhizal plants have current and potential applications in reclaiming and conditioning soil, producing timber and pulp, and acting as nurse, windbreak, ornamental, and fuelwood plants (73, 76). Globally, they have potential for integrating into schemes for addressing issues of pyrodenitrification (70) and reforestation (12, 76).

TABLE 1. Actinorhizal plant genera and *Frankia* isolates

Family and genus	Isolates[a]	References[b]
Betulaceae		
Alnus	+, I, E	14, 17, 19–21, 31, 45, 59, 85, 97, 109, 128, 188, 194, 201
Casuarinaceae		
Allocasuarina	+, I, E	294
Casuarina	+, I, E	17, 66, 78, 79, 293
Gymnostoma	+, I, E	202
Ceuthostoma	–	
Coriariaceae		
Coriaria	+, N	66, 164, 165
Datiscaceae		
Datisca	+, N	66, 165
Elaeagnaceae		
Elaeagnus	+, I, E	14, 16, 21, 81, 128
Hippophaë	+, I, E	66, 85, 94
Shepherdia	+, I, E	14, 81
Myricacea		
Comptonia	+, I, E	62, 143
Myrica	+, I, E	17, 55, 59, 139, 249
Rhamnaceae		
Ceanothus	+, N	22, 143
Colletia	+, N	3, 60, 65, 95
Discaria	+, N	63a, 149
Kentrothamnus	–	
Retanilla	+, N	63a, 149
Talguenea	+, ?	63a
Trevoa	+, N	64
Rosaceae		
Cercocarpus	+, N	22
Chamaebatia	–	
Cowania	+, ?	17
Dryas	–	
Purshia	+, N	17

[a] Symbols: –, isolates not reported; +, isolates obtained; I, infective; N, noninfective; E, effective in fixing N_2; ?, infectiveness unknown or unreported.

[b] Numerous other isolates have been made from members of some genera; references provided are the earliest and/or most representative reports. Additional isolates have been obtained from some genera but have been reported informally.

DESCRIPTION OF THE GENUS AND TAXONOMIC CONSIDERATIONS

General Description

The identity of the actinorhizal root nodule inhabitants as actinomycetes was established in 1964, when electron microscopy revealed the prokaryotic structure of the microorganisms in *Alnus glutinosa* and *Myrica cerifera* root nodules (26, 230). A more detailed description became available only in 1978, when the isolation of strain CpI1 (now known as HFPCpI1) from *Comptonia peregrina* nodules was reported (62) and the ability of the organism to reinfect the host plant and reestablish the symbiosis was confirmed (125). Once the unique morphology of cultured *Frankia* strains had been described, it became evident that a strain isolated from *A. glutinosa* nodules by Pommer in 1959 strongly resembled HFPCpI1 (194). Pommer's strain was subsequently lost, but among the many claims for isolating the nonleguminous symbiont prior to 1978, his alone has become accepted.

Frankia strains are readily recognized by their appearance in liquid culture. They form extensive hyphae and bear sporangiospores in multilocular sporangia located either terminally or in an intercalary position on the hyphae (Fig. 1

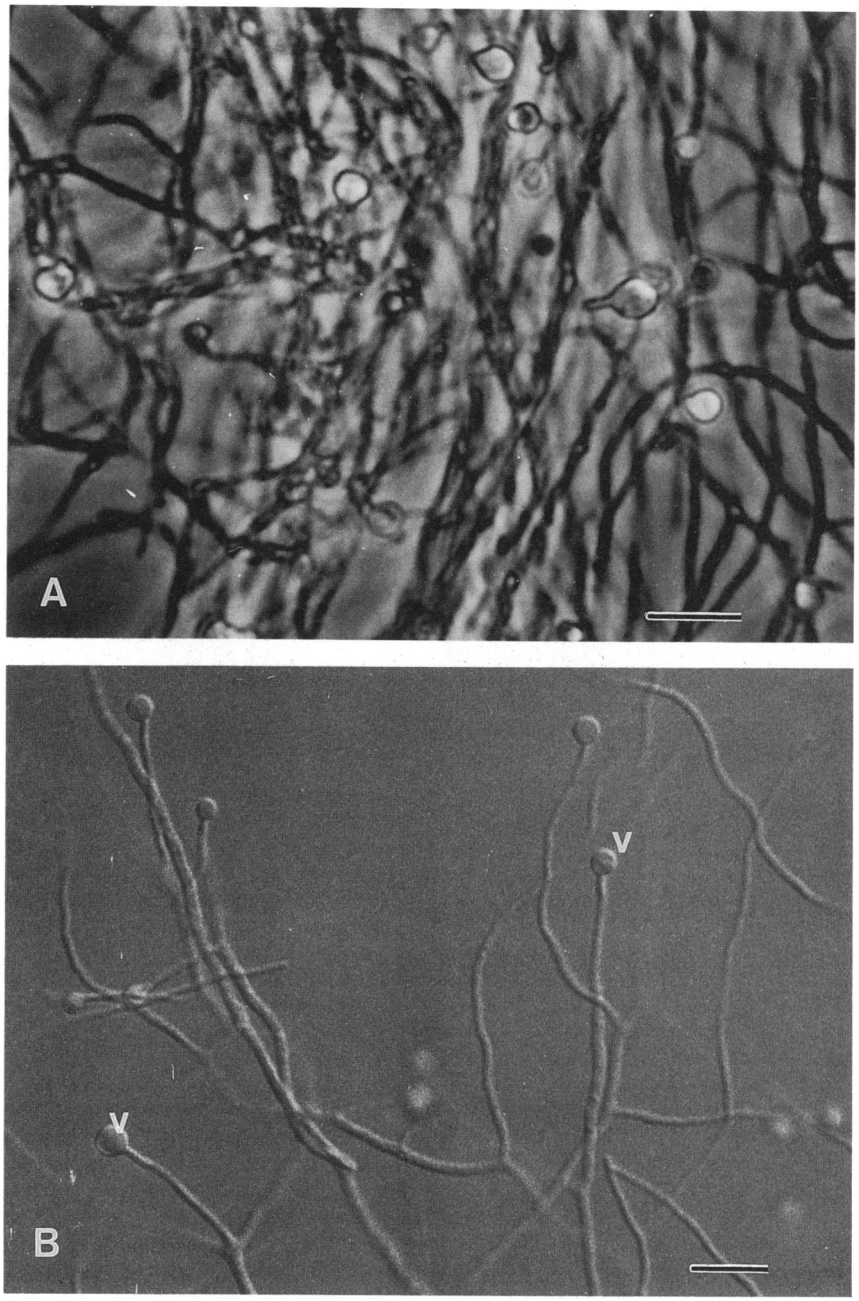

FIG. 1. Light-microscopic views of *Frankia* cultures as they appear during N_2 fixation. (A) Phase-contrast photomicrograph of *F. alni* HFPCpI1 during N_2 fixation. The phase-bright structures are the vesicles. (B) Nomarski optics photomicrograph of *Frankia* sp. strain CcI3 during N_2 fixation. Note the vesicles (v) formed on the tips of hyphal branches. Bars, 5 μm.

and 2). Aerial hyphae are not produced on solid media. A distinguishing characteristic of *Frankia* strains (with some exceptions [see reference 165]) is the differentiation of vesicles in culture and often in symbiosis (Fig. 1). Vesicles are lipid-encapsulated, roughly spherical structures measuring between 2 and 6 μm in diameter, attached either terminally or laterally to hyphae by a short vesicle stalk that is also encapsulated; they are commonly made in response to nitrogen deprivation. Their development in culture and symbiosis and their participation in N_2 fixation are discussed in more detail below.

Some members of the genus produce pigments in culture; red, yellow, orange, pink, brown, greenish, and black pigments have been reported, depending on the strain, the medium used, and the age of the culture (142). A common red pigment has been identified as 2-methyl-4,7,9,12-tetrahydroxy-5,6-dihydrobenzo[*a*]naphthacene-8,13-dione and a carboxylated derivative (96). Since pigmentation is highly variable, it is a poor criterion for placement into the genus but may prove useful as a phenotypic trait for species recognition.

Chemotaxonomic studies have revealed that all *Frankia*

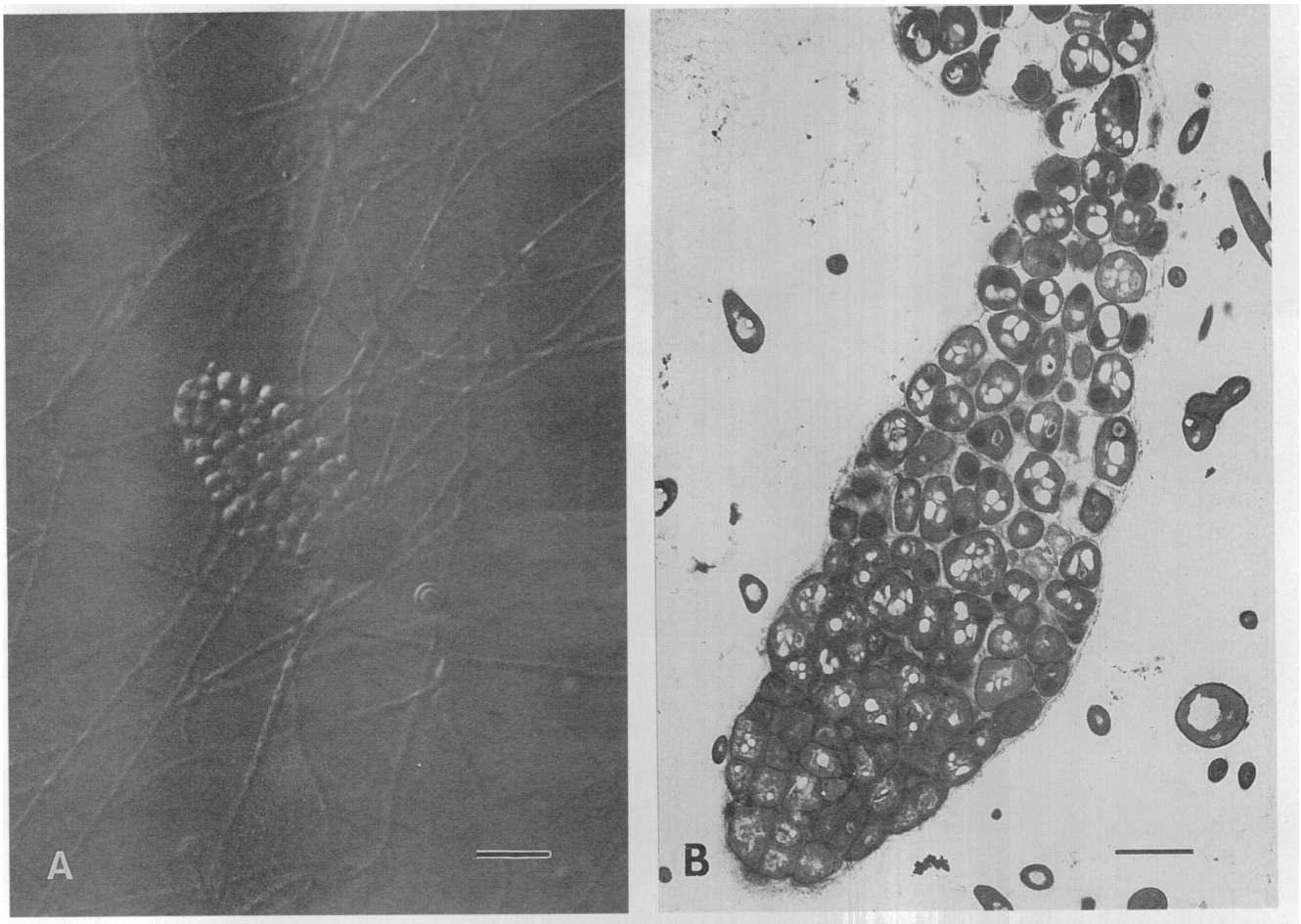

FIG. 2. Nomarski optics (A) and transmission electron (B) photomicrographs of *Frankia* multilocular sporangia from *Casuarina* strain CcI3 (panel A) and an uncataloged *C. equisetifolia* strain (panel B). In both cases, more mature spores are at the top and less mature spores are at the bottom. Bars, 5 μm (panel A) and 2 μm (panel B). Panel B courtesy of R. H. Berg.

strains tested have a type III cell wall containing *meso*-diaminopimelic acid, alanine, glutamic acid, muramic acid, and glucosamine (142). This wall type is common among the aerobic actinomycetes. Whole-cell sugars include variable amounts of fucose, ribose, xylose, madurose, mannose, galactose, glucose, rhamnose, and 2-*O*-methyl-D-mannose (142, 168, 248). St.-Laurent et al. (248) considered the presence of 2-*O*-methyl-D-mannose to be diagnostic for the genus *Frankia* and used its relative abundance to discriminate between strains belonging to the *Alnus* host specificity group (including *Myrica* and *Comptonia* species) and strains infective on members of the family Elaeagnaceae (*Elaeagnus*, *Hippophaë*, and *Shepherdia* species).

Phospholipid analysis places *Frankia* strains into group PI, containing phosphatidylinositol, phosphatidylinositol mannosides, and diphosphatidylglycerol; nitrogen-containing phospholipids are absent (141). This grouping distinguishes them from members of the genus *Geodermatophilus* (group PII), which also produce multilocular sporangia and are currently the closest known relatives of *Frankia* strains (99). Fatty acid analysis has been used both to classify "atypical" strains into the genus (165) and to discern subgroups among existing members (237). Although both of these objectives can be achieved by using appropriate control strains, no clear taxonomic marker that would place an unknown strain within the genus has been identified. The discovery of hopanoids in some *Frankia* strains may provide an additional chemotaxonomic marker in the near future (48). In the few strains that have been analyzed, a menaquinone pattern consisting mainly of MK9(H_4) (menaquinone with nine isoprene units, four of which are hydrogenated) with lesser amounts of MK9(H_6) and MK9(H_8) was found (138). Again, such a pattern is common among the aerobic actinomycetes.

The G+C content of more than 45 *Frankia* strains studied, isolated from a variety of plants, falls in the relatively narrow range of 66 to 75 mol%. This high mol% G+C places *Frankia* strains among the euactinomycetes and constitutes one of the fundamental prerequisites for belonging to the genus (11, 87), although the relative uniformity of the values renders the G+C content of little use in distinguishing species among *Frankia* strains.

As minimum criteria for including strains within the genus, it is generally agreed that the combination of morphology in culture and isolation from an actinorhizal root nodule is sufficient (142). A high G+C content, cell wall type III, a phospholipid pattern of PI, and, increasingly, rRNA sequences are all additional criteria used to place new strains in the genus *Frankia*.

Nomenclature

The description of HFPCpI1 provided investigators with the key information needed to isolate and recognize *Frankia* strains. As a result, hundreds of isolates were obtained from a variety of plants in a relatively short time during the late 1970s and early 1980s. This harvest of isolates proved both boon and bane to researchers in the field, since no framework existed for naming, grouping, or identifying isolates as they became available. The original HFPCpI1 isolate was so named because it was "*Comptonia peregrina* isolate number 1" (62). Additional strains from other plants were similarly named (for example, ArI3 from *A. rubra* [45], AirI1 and AirI2 from *A. incana* subsp. *rugosa* [137], and $EAN1_{pec}$ from *Elaeagnus angustifolia* [128]). Many of the first strains to be isolated are still referred to by these trivial designations. However, since additional strains from a single plant species were isolated by different laboratories, a more coherent cataloging system became necessary. In 1983, a numbering system based on the laboratory of origin, host genus, host species, and a six-digit number unique to the strain was established to help bring order to the proliferation of strains in collections around the world (15, 135, 136). This consensus cataloging system is still used for ordering strains in collections (141).

The genus name *Frankia* was proposed in 1886 by J. Brunchorst to honor his mentor, A. B. Frank, a Swiss microbiologist (198). Since Brunchorst and Frank considered the microorganism to be a fungus, the name was not generally used until Becking, in 1970, revived it and redefined the genus as consisting of members of the *Actinomycetales* in a new family, *Frankiaceae* (24). Becking further proposed that 10 species be established (*Frankia alni*, *F. elaeagni*, *F. brunchorstii*, *F. discariae*, *F. casuarinae*, *F. ceanothi*, *F. coriariae*, *F. dryadis*, *F. purshiae*, and *F. cercocarpi*) based on the source plant, and, since numerous attempts to obtain the organisms had failed, he described the members as "obligate symbiotic organisms."

Frankia strains still constitute a separate family within the order *Actinomycetales*. In *Bergey's Manual of Systematic Bacteriology*, vol. 4 (1989), they are included among "actinomycetes with multilocular sporangia" (141). Other genera in this group include *Geodermatophilus* and *Dermatophilus*. Recent 16S rRNA sequence analyses of representatives of these genera have revealed a relatively close phylogenetic relationship between *Frankia* and *Geodermatophilus* but not *Dermatophilus*; an invalidly described isolate from the Black Sea called "*Blastococcus*" also clustered with *Frankia* (99; see below) (Fig. 3). Thus, future descriptions of the family *Frankiaceae* should include members of these other genera. The family *Dermatophilaceae* will consequently contain only the genus *Dermatophilus* (99).

Grouping of *Frankia* Strains into Species

Although the genus designation *Frankia* and the creation of the family *Frankiaceae* have been unanimously accepted as described by Lechevalier and Lechevalier (141), the species assignations according to host plant of origin have proven more controversial (141). The difficulties encountered include the observations that individual strains often nodulate plants from different plant orders; strains from the same "genomic species" have been isolated from members of different plant families; some strains cannot reinfect their source plant; and unpredictable nodulating abilities have been observed, especially among the so-called *Elaeagnus*

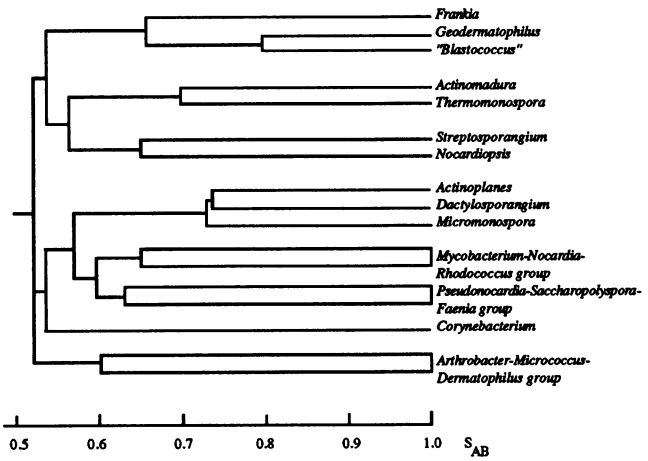

FIG. 3. Phylogenetic placement of the genus *Frankia*. Adapted from reference 99 with permission. S_{AB}, similarity coefficient.

strains (10, 22, 57, 87). Nevertheless, a considerable degree of host plant specificity does occur, with the result that the host plant origin and the ability to nodulate within certain host specificity groups are relevant but not determinative characteristics for strain identification or classification.

Arranging *Frankia* strains into phenotypically related groups have proven to be a difficult task. Classical physiological testing is of little use in grouping *Frankia* strains since they all grow slowly (doubling times of 15 to 48 h or more) and the results often vary depending on how long the strains are allowed to grow. Consequently, several other approaches have been taken with various degrees of success. Early attempts to correlate serological groups with host range or other phenotypic parameters were largely abandoned when such correlations proved more complex than was initially thought (18, 137). Additional approaches to grouping strains have included comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins (34, 37, 92), analyses of whole-cell sugar patterns (168, 248), fatty acid analysis (165, 237), isoenzyme analysis (91, 197), DNA restriction pattern analysis of total DNA (56), *nif* gene restriction fragment length polymorphism analysis (182, 190), DNA-DNA homology studies (2, 10, 56, 87), and low-frequency restriction fragment analysis (52). Each of these approaches has yielded phenotypic and in some cases genotypic subgroups within host specificity groups.

Not all approaches have been taken with classification in mind; some have been used primarily to assess the diversity of strains isolated from an individual plant species or of strains obtained from a limited geographical area (34, 37, 56, 92). Of the techniques used, only DNA-DNA homology and perhaps the recently developed restriction fragment length polymorphism can unequivocally place an unknown strain within a genospecies. The other techniques yield well-defined groups independent of phylogenetic relationships, but the sensitivity of the methods remains unknown. As additional chemotaxonomic and phylogenetic data accumulate in the present polyphasic approach to *Frankia* taxonomy, the structure of the genus *Frankia* will become increasingly clear (172).

The available biochemical, physiological, and phylogenetic information on certain well-studied groups of strains has rekindled the desire to establish *Frankia* species. To do so, it is desirable to satisfy the consensus that new geno-

species be defined in phylogenetic terms on the basis of DNA sequence data (286). Thus, DNA-DNA reassociation kinetics that approximate 70% or greater DNA relatedness, together with a 5°C or less ΔT_m between strains, would support placement of two strains in the same species, with the caveat that any genospecies so identified should not be named until it is distinguishable from another genospecies on the basis of phenotypic properties. For *Frankia* strains, sufficient information is available to support the species identification of some members of the genus.

Lalonde et al. proposed the acceptance of the species *F. alni* and *F. elaeagni* and created *F. alni* subsp. *pommerii* and *F. alni* subsp. *vandijkii* (132). This proposal was based on the results of biochemical and physiological studies done on strains isolated mainly from members of the families Betulaceae, Myricaceae, Elaeagnaceae, and Casuarinaceae. Since many of the studies used the same strains (91, 92, 237, 248), the consistency of the resulting groups could be compared. However, at the time, DNA-DNA reassociation analyses had been done on only a few of the strains subjected to other types of analyses, and acceptance of the proposed species designations was not universal. A more extensive DNA relatedness study that includes strains grouped by other methods has since been done. Fernandez et al. (87) identified nine genomic species among 43 isolates examined. Three genomic species were found among the strains that infect *Alnus* species, five were found among strains that infect members of the family Elaeagnaceae, and one contained 11 strains isolated from members of the Casuarinaceae. Nine strains (four isolated from *Alnus* species, two isolated from members of the Elaeagnaceae, and three isolated from members of the Casuarinaceae) were not classified and may represent additional genomic species. Other DNA-DNA homology studies with different strain collections have also confirmed the overall diversity of *Frankia* strains suggested by other methods (2, 10, 56).

The largest group of *Alnus*-infecting strains, genomic species 1, was first established as genogroup I by An et al. in their early DNA homology work (10). They established genogroup I with seven isolates from *Alnus* species plus one from *Comptonia peregrina* and one from *Myrica pensylvanica*, with DNA from ArI4 (from *A. rubra*) as a reference. The coherence of this group has since been confirmed by numerous phenotypic and genetic analyses and thus has a valid claim to the species epithet *F. alni* as described by Lalonde et al. (132) with strain HFPCpI1 as the type strain. The proposal that the subspecies *F. alni* subsp. *vandijkii* and *F. alni* subsp. *pommerii* be established has generated some confusion in the naming of strains. As Beyazova and Lechevalier (52) have pointed out, a subspecies of *F. alni* should be *alni*. Also, strain ARgP5^{Ag} (ULQ0132105009), which is the type strain for the *F. alni* subsp. *vandijkii* group of Lalonde et al. (132), was in genomic species 3 in the analysis of Fernandez et al. (87) and had only a 2% DNA homology with members of genomic species 1, which contained HFPCpI1. Therefore, the proposal that *vandijkii* be considered a subspecies of *F. alni* is problematical, since a subspecies should be genetically close to but phenotypically distinct from the type species (286). Because of the results from the DNA-DNA reassociation studies, the definition of the subspecies *vandijkii* and *pommerii* needs to be reconciled with current knowledge.

The impression from early studies that *Elaeagnus* strains are highly diverse in their physiology, morphology, and biochemistry has been confirmed by DNA-DNA reassociation studies (10, 87). An et al. (10) found no genogroup among 10 isolates (5 from *Elaeagnus* species, two from *Casuarina equisetifolia* and one each from *Ceanothus americanus*, *Purshia tridentata*, and *Alnus incana* subsp. *rugosa*) tested against DNA from EuI1a (from *Elaeagnus umbellata*) or ArI4. The 13 Elaeagnaceae strains tested by Fernandez et al. (87) constituted their genomic species 4 through 8. Genomic species 4 had six members, species 5 had three, and species 6, 7, and 8 had one each. Unfortunately, the type species proposed for *F. elaeagnii*, strain SCN10a (ULQ190201001) (132), was not studied, so its relationship to other genomic species remains unknown.

A surprising degree of similarity has been found among strains that were originally isolated from members of the Casuarinaceae. Of the 11 strains tested by Fernandez et al. (87), 8 constituted genomic species 9, with percent reassociations ranging from 69 to 100%. The three strains that remained unclassified were "atypical," since they failed to reinfect the source plant but could infect members of the Elaeagnaceae. The coherence of the genomic species 9 group may be surprising considering that the strains were isolated from nodules collected at geographically separated locales, including Senegal, Australia, Florida, Brazil, Thailand, and Madagascar. However, as Fernandez et al. pointed out (87), all casuarinas originated from Australia and the South Pacific Islands and have only recently been transplanted to other regions for various purposes; therefore, the relative coherence of the group may reflect their limited geographical origin. Alternatively, the strains in culture simply may be the more easily isolated or faster-growing strains and are therefore not representative of the true diversity in the field. In any event, the strains in genomic species 9 isolated from *Casuarina* species may be sufficiently defined both genetically and physiologically to warrant the creation of a new species name.

From the work done on grouping *Frankia* strains, it is clear that the true diversity of the genus is considerably greater than was initially suspected. This fact may require that the genus ultimately be split into two or more genera. In a sense, the definition of what constitutes members of the genus is biased by the relative ease with which some isolates can be obtained and the difficulties encountered in isolating other strains, so our view of a typical *Frankia* strain tends to correspond to characteristics expected, for example, in an isolate from an *Alnus* or *Elaeagnus* host. Considering that, of the eight plant families whose members are infected by *Frankia* strains, infective isolates are readily obtained from members of only four (Betulaceae, Casuarinaceae, Elaeagnaceae, and Myricaceae) and that only noninfective isolates, atypical isolates (unable to reinfect the source plant species), or no isolates have been obtained from members of the other families, the diversity of *Frankia* strains is no doubt greater than predicted on the basis of the limited sampling done thus far. Newer approaches using rDNA amplified directly from the nodule, circumventing the need for cultivation, should provide a clearer picture of the diversity of strains that remain uncultured (164, 178). However, the temptation to be satisfied with only genetic or phylogenetic means to describe these slow-growing actinomycetes should be avoided; physiological work may proceed slowly, but it will surely tell us more about the biology of the symbiosis in the long run.

Phylogenetic Position of *Frankia*

On the basis of sporangium morphogenesis, Lechevalier and Lechevalier (139) suggested that *Frankia* strains were

likely to be related to members of the *Dermatophilaceae* within the *Actinomycetales*. This proposal has proven to be substantially correct in subsequent studies on 16S rRNA and rDNA sequencing and cataloging (89, 99, 178). Such techniques place *Frankia* and *Geodermatophilus* in a separate line of descent within the actinomycete lineage, among the high mol% G+C bacteria (89) (Fig. 3). The results of a nearly complete reverse transcriptase sequencing of the 16S rRNA from *Frankia* strain Ag45/Mut15 agree with the oligonucleotide cataloging method (99). A complete sequence of the 16S, 23S, and 5S rRNA operon in *Frankia* strain ORS020606 from *Casuarina* species has recently been published; the 16S sequence was shown to be 95% similar to the Ag45/Mut15 sequence and 90% similar to the *Streptomyces ambofaciens* sequence (185).

In an attempt to establish phylogenetic relationships between the *Frankia* genomic species, Nazaret et al. (178) compared sequences of a 268-bp fragment of 16S rDNA amplified from 35 strains, including one unisolable strain in an alder nodule. With one exception, strains within a genomic species as defined by Fernandez et al. (87) had a unique sequence. The exception was between representatives of genomic species 4 and 5, which had identical rDNA sequences but only 38 to 49% DNA-DNA homology (87). In studies of other organisms, 16S rRNA sequences have sometimes predicted that two organisms should belong to the same species but DNA-DNA hybridization results and phenotypic traits placed them in separate species (90). This conflict apparently arises when significant DNA-DNA homology exists but at a level somewhat below the 70% benchmark value accepted as defining a species boundary, as in the case of genomic species 4 and 5 (90). Thus, identical or very similar short stretches of rDNA do not always reflect DNA-DNA hybridization results (90). Analysis of the *Frankia* sequences suggested that all of the strains isolated from *Elaeagnus* species and some of the atypical strains isolated from *Casuarina* species formed a separate, relatively well-defined group, whereas other atypical strains isolated from *Casuarina* species grouped with strains, from *Casuarina* species, that constitute genomic species 9. These latter strains, in turn, grouped closer to *F. alni* (genomic species 1) than to the strains, isolated from *Alnus* hosts, in genomic species 2 and 3. Thus, the impression gained from these studies is that strains isolated from *Alnus* species are more deeply divided than those from *Elaeagnus* species. Again, however, this result must be interpreted in light of the short sequence used and the limited number of strains tested. In addition, the set of strains from alder was selected from a much larger pool of strains than the strains from *Casuarina* or *Elaeagnus* species; this may have led to the selection of a wider diversity of strains from alder. Further studies will be necessary as more strains become available.

Other semantides used to place *Frankia* in phylogenetic schemes include the Fe-protein (*nifH*) and the α subunit of the MoFe protein (*nifD*) of the nitrogenase complex, and glutamine synthetases I (*glnA*) and II (*glnII*) (122, 184, 187, 190, 271). Studies on the *nifH* genes and their amino acid sequences placed *Frankia* closer to *Anabaena* and the gram-negative proteobacteria than to the low-G+C gram-positive organisms such as *Clostridium* (184). Since this placement was at odds with 16S rRNA studies, the results were interpreted as possibly reflecting a recent lateral gene transfer from an ancestral gram-negative organism to both *Anabaena* and the progenitor of *Frankia*. Subsequent analysis of the *F. alni* ArI3 *nifD* gene gave a similar relationship within statistical fluctuation and was interpreted as ". . .evidence of the survival of different gene duplicates in two lineages of Gram positive bacteria: *Frankia* and *Clostridium*" (187), thus only giving the impression of a phylogenetic split in the gram-positive bacteria. Recent studies on glutamine synthetase genes (*glnA* and *glnII*) from HFPCpI1 have revealed that the *Frankia glnA* sequence is closest to *Streptomyces*, then to *Anabaena*, and then to gram-negative organisms but again relatively far removed from the low-G+C clostridia and bacilli (122). Thus, a possibility to consider is that actinomycetes may have more of an affinity with cyanobacteria than is revealed by the deep branches within the eubacterial phylogenetic trees created by 16S rRNA cataloging methods (89).

STRUCTURE AND ULTRASTRUCTURE

General Structure

When grown on most media, frankiae are characterized by three structural forms, hyphae, sporangia, and vesicles (Fig. 1 and 2). The hyphae are septate and often tightly interwoven in culture; in all strains studied, they produce either terminal or intercalary multilocular sporangia. Vesicles are normally produced in culture on nitrogen-free or nitrogen-poor media (259, 292) and are the sites of nitrogenase. In symbiosis, the host plant obviously plays a significant role in modifying *Frankia* morphology since there is a large variation in the presence and absence of sporangia and in the size, shape, or presence of vesicles.

The structure of *Frankia* cells both in vitro and in symbiosis was extensively reviewed by Newcomb and Wood in 1987 (181); since then, there have been some significant developments, particularly in our understanding of vesicle structure. *Frankia* cells have been difficult to study with the electron microscope because of the delicate nature of some components of the cells, in particular the extracellular envelope of vesicles. Some of the difficulties have been resolved by freeze substitution and freeze fracture techniques, but there still remain some elusive problems in analyzing structure from chemically fixed specimens (181).

Free-living *Frankia* cells

Hyphae. Free-living cells under the light microscope show branched septate hyphae ranging in width from 0.5 to 1.5 μm with a large number of bright areas when seen under phase-contrast and dark-field optics. Cell walls in chemically fixed (CF) material appear to be composed of two layers of electron-dense material, a base layer and an outer layer (16, 110, 126, 134). Cross-walls originate from the base layer. Membranous layers are sometimes visualized outside the outer wall layer (110, 180, 181), and the cell wall may also contain spherical or ovoid inclusions in both CF and freeze-substituted (FS) material (134).

Internally, hyphal cells contain numerous rosette-shaped granules, which are presumed to be glycogen (36, 134) and lipid droplets. Small, round, clear areas are seen in FS cells, and there is no indication of a condensed nucleoid region, which is observed in CF cells (134). The clear areas seen in FS cells may have contained lipid, since this is not retained in FS cells (134). Individual ribosomes and polyribosomes are seen in FS hyphal cells as relatively large (300-nm) granular bodies (134). Around the periphery of the FS hyphal cell cytoplasm are large numbers of cytoplasmic tubules, circular in cross section and averaging 45 nm in diameter. These structures underlie the cell membrane both at the cell septum and at the outside wall (134).

An extracellular multilayered envelope was first identified in hyphae of free-living *F. alni* HFPCpI1 (180). This envelope has been much studied on vesicles (see below), but its presence on hyphae is still the subject of debate. The envelope is removed by normal chemical fixation (181) and by freeze substitution techniques (134), but the fact that multilayered envelopes have been seen on hyphae of symbiotic *Frankia* cells (1, 41, 131) indicates that lipid enveloped hyphae may be common among the frankiae.

Sporangia. Before embarking on a description of the structure of spores and sporangia, it is necessary to describe sporulation both in culture and in symbiosis. Sporangia were first identified unequivocally in the nodules of *Alnus glutinosa*, before *Frankia* strains became available and despite a wealth of prior observations and misidentifications (280). Confusion over the nature of the spore-like structures in root nodules was put to rest with the isolation of *Frankia* strain HFPCpI1, which produced multilocular sporangia that appeared unique to the *Frankia* group.

Sporangia are produced readily in culture by all *Frankia* strains isolated, and spores as infective propagules are 1,000 times more effective at nodule production than are equal volumes of hyphae (61). The enigma of sporulation is not due to the behavior of *Frankia* in culture, which is consistent, but is due to the fact that in symbiosis nodules may be sp^+ (contain spores) or sp^- (lack spores) apparently independent of their performance in culture. Recent reviews of this subject cover the history of our understanding of the phenomenon, and only a short summary will be given here (222, 265).

Sporulation in nodules is well documented only for *Myrica* and *Alnus* species and, even in these, is sporadic and somewhat site specific (222, 265). Sporulation in nodules of other genera is not well studied. Evidence for a genetic, as contrasted with an environmental, basis for sporulation in nodules is provided by the observation that nodule suspensions from sp^+ and sp^- nodules, when used as inocula, produce sp^+ and sp^- nodules, respectively, in cultured plants (115, 166, 274–276). These results support the concept, developed by Van Dijk (277, 278), that there are two genetically distinct bacterial types based on their expression of sporulation in root nodules, which is independent of their sporulation ability in culture.

Whether genetically distinct sp^+ strains actually exist is still matter for debate, since it is widely believed that no sp^+ strains are currently in culture. Strains that have been cultured from sp^+ nodules are very difficult to grow (188) and are often ineffective on the host from which they had been isolated (16, 100). In one case (16) a culture (EuI1b) was obtained from an *Elaeagnus* sp^+ host and produced copious sporangia in culture and in symbiosis with *Elaeagnus* species but failed to produce vesicles in the nodules and was thus ineffective for N_2 fixation.

The enigma of sporulation in nodules is unresolved. On the one hand there is strong evidence to support the theory that two distinct races of *Frankia* exist which control their sporulation in symbiosis but not in culture (115, 188, 279). On the other hand, the host and the environment in which the host lives have a strong effect on sporulation, both on the presence and absence of sporangia and on the seasonal periodicity of spore production (274–276). A recent attempt to resolve this dilemma led to the postulate that if stable strains could be cultured from sp^+ nodules and reinfected to produce effective nodules, added weight would be given to the existence of sp^+ strains (204). Strains recently isolated from *Casuarina* and *Myrica* sp^+ nodules do show this potential, although the sporangia are sparse and normally seen only under the electron microscope (204). It appears likely that as further strains are isolated from sp^+ nodules, or perhaps as polymerase chain reaction (PCR) amplification of rRNA genes from whole nodules becomes more widely used, the differences between sporulation ability may be resolved into a genetic divergence. It is also too early to rule out the possibility that genetically sp^+ strains exist and that other strains respond to environmental signals to express the sp^+ phenotype in symbiosis.

In addition to the potential of different strains to sporulate in nodules, there is a growing body of evidence to suggest that sp^+ and sp^- nodules have distinct physiological differences. Work on *Alnus* (155, 188, 238, 287), *Comptonia* (275), and *Myrica* (166) species indicates that plants inoculated with sp^- cultures produce significantly more biomass than those inoculated with sp^+ nodule suspensions do. Further, the work on *Myrica* species shows that as well as producing more biomass on host plants, sp^- nodules have higher specific activity and lower energy cost of nitrogenase (166).

There are many reports which show that sp^+ nodule suspensions and spores produced in culture are much more infective than nodules or cultures without spores (61, 277). The conditions for spore germination have been investigated (273); they show that many strains have low spore germination potential and that this is correlated with failure to release spores from sporangia in culture. Strains which release spores show good germination in culture (273) and, coincidentally perhaps, also show low levels of sporulation in host plants (204). Recent studies have shed some light on the problem of spore germination in culture, with the observation that certain types of agar inhibit germination (143a).

The problem of spores, sporulation, and infection is far from resolved. The role of spores in infection is, as yet, determined only from indirect experimentation, and the possible stimulus of spore germination by host plants is a matter very much open to direct study (273).

Sporangial structure. Sporangia develop as terminal or intercalary structures (180). Segmentation within the enlarging sporangia produces a multilocular sporangium containing many spores (Fig. 2). CF material shows the developing spores with electron-translucent nucleoid regions with dispersed fibrils and numerous lipid droplets. Like the hyphae, CF external walls often show the residue of a laminate envelope (134). As with hyphae, the nucleoid region is not evident after FS preparation (134), with the cytoplasm of the developing sporangia dispersed like that of the hyphae (134). The mature spores show evenly dispersed cytoplasm, but the tubules, which are such a prominent feature of hyphae, are not present in the developed spores (134).

In the few strains studied, including strain AirI2 isolated from *Alnus incana* subsp. *rugosa* (110), strain ArI3 from *Alnus rubra* (110), strain HFPCcI3 from *Casuarina* species (134), strain EuI1 from *Elaeagnus* species (16), and strain HFPCpI1 from *Comptonia peregrina* (180), sporangia develop by hyphal thickening and then by segmentation by septa originating from the inner layer of a double-layered sporangial cell wall (110); this type of sporogenesis has been termed enterothallic by analogy with spore development in fungi (148). The result of such a pattern of development is that older, more loosely packed spores are distal to younger, more densely packed, developing spores. The intersporal matrix appears to originate from the inner layer of the cell wall. The younger spores are somewhat irregularly shaped, and older spores are spherical to ovoid and measure about 1 to 5 μm in diameter. They are nonmotile, unlike those found

in the multilocular sporangia from the related genus *Geodermatophilus* and the less related *Dermatophilus*, whose members produce spores that may bear tufts of flagellae. The number of spores per sporangium ranges from a few to several hundred depending on the age and nutrition of particular strains.

Vesicles. The vesicles are, without doubt, the most definitive structure characterizing the genus (Fig. 1). Although functionally analogous in many ways to heterocysts of cyanobacteria, the vesicle is a unique developmental structure designed for physiological compartmentation and has not been described for any other prokaryotic group. Vesicles are normally initiated only when the nitrogen source in the medium is withdrawn or during growth on certain nitrogen sources, such as some amino acids, that cannot be degraded to ammonia (see below); they initially develop as terminal swellings on hyphae or on short side branches. These early structures are separated by a septum near the base and are termed provesicles (88). Provesicles are spherical, phase-dark cells, 1.5 to 2.0 μm in diameter, with dense cytoplasm, and they may show the initiation of internal compartmentation; no nitrogenase activity is associated with provesicles (88). Although vesicle formation is controlled largely by nitrogen supply, some *Frankia* strains continue to form vesicles even in the presence of NH_4^+, although such strains are certainly the exceptions (93, 158).

Provesicles rapidly develop into mature vesicles (2 to 4 μm in diameter), which are distinctly phase bright (88) and also show birefringence under polarized-light microscopy (266) and a bright halo under dark-field microscopy (192). The development into a mature vesicle is accompanied by a number of complex structural changes which correlate very strongly with the appearance of nitrogenase activity (88). Mature vesicles are characterized by two important structural elements, the vesicle envelope and internal septation, both of which appear to be necessary prerequisites for nitrogenase activity in cultured *Frankia* cells but neither of which appears essential in symbioses.

The vesicle envelope was first analyzed in detail in 1982 by Torrey and Callaham (266), who observed that enveloped vesicles showed a marked birefringence under polarized light. They interpreted this result to imply the presence of a highly structured laminated layer, which they showed by elegant freeze-fracture techniques to be a laminate of lipid monolayers. These layers appear to be totally lost in normal fixation procedures, leaving a space around the vesicle (a "void area" [131] [see Fig. 6]) where, presumably, the lipid layers have been solubilized. Each layer was estimated to be about 4 nm thick (266). The lipid nature of the envelope has been confirmed by the use of the fluorescent dye Nile red (133). Further structural studies of the envelope have been facilitated by the development of techniques to isolate intact vesicles (183, 220) and vesicle envelopes (104) from N_2-fixing *Frankia* cells. The multilayered envelope can be stripped off by sonication and stained with permanganate to visualize the distinct multilaminate structure (Fig. 4). The envelope is not uniformly distributed around the vesicle, with more laminations appearing in the vesicle stem region than in the distal regions. In addition, the number of layers is correlated with the pO_2 of the surrounding medium (192; also see below).

The relative lipid content of vesicles and hyphae was analyzed by comparing the lipids of isolated vesicles with those of hyphal cultures (270). This analysis showed that vesicles have a higher proportion of neutral lipids and glycolipids but lower levels of polar lipids than vegetative cells. Analysis of the neutral-lipid fraction revealed the presence of abundant unidentified compounds which were suggested to be long-chain fatty acids or alcohols. It now becomes evident that *Frankia* cells contain a very high concentration of the pentacyclic hopanoid lipid bacteriohopanetetrol (48). Further work on isolated vesicle envelopes from *F. alni* HFPCpI1 indicates that they consist mainly of bacteriohopanetetrol and bacteriohopanetetrol phenylacetate monoester in approximately equal amounts (Fig. 5) (46). Bacteriohopanes are common in many bacterial groups (213) and have been proposed to play a direct role in membrane stability and fluidity (75, 121). Their presence in the vesicle envelopes has a number of interesting implications for O_2 protection, discussed more fully below.

The outer laminate envelope extends down the stalk of the vesicle past the basal septum which separates the vesicle from the hypha (134). The true wall is separated from the envelope by a small gap in FS material and by a large space artifact in CF material (134). The internal structure of vesicles is characterized by a number of internal septations, which subdivide the mature cell. Ribosomes are frequent, as are the very striking bundles of microfilaments exclusive to vesicles, which are closely associated with the septa (134).

Vesicles appear to have a limited life (88), although this has not been carefully investigated by using continuous or semicontinuous culture techniques. During senescence, internal septa begin to become irregular, nitrogenase activity is lost, and they become ghosts which are recognizable in culture as deformed, thin-walled, nonfunctioning cells (60, 88). Although the main physiological significance of vesicles is their nitrogenase function, a proportion of vesicles have also been observed to "germinate" and produce hyphae in culture (219). Both isolated vesicles and those still attached to parental hyphae can grow through the vesicle envelope or from the vesicle stem. The potential to regrow may have some significance after liberation from vesicle-containing nodules in the field.

Symbiotic *Frankia* Cells

Introduction. The structure of actinorhizal nodules and symbiotic *Frankia* cells has been reviewed in detail (181), and readers are referred to reference 181 for an analysis of symbiotic structure. What follows is a brief description of the diversity of forms that *Frankia* cells and the host plant may assume in symbiosis.

Nodule morphology and anatomy. The nodules produced by *Frankia* strains on actinorhizal plants differ markedly from those initiated by *Rhizobium* strains on legumes. These differences occur because the bacterial symbionts, and the host plants themselves, are extremely diverse (233). The infected zone of legume nodules is central and is normally contained within an endodermis and an inner cortical layer of tightly packed cells, with the vascular tissue lying outside these tissues. In contrast, actinorhizal nodules normally have a central stele that has infected tissue adjacent to it or around it.

Infection process. *Frankia* cells enter symbiosis by root hair infection (for *Alnus*, *Casuarina*, *Comptonia*, and *Myrica* species [51, 63, 264]) or by a process of intercellular penetration of root epidermis and cortex (for *Ceanothus*, *Elaeagnus*, and *Shepherdia* species [146, 161, 203]).

Root hair infection is characterized by root hair branching and curling. Only one root hair infection is required for nodulation (47), and where multiple root hairs are infected, cortical infection is related to only one root hair infection (63). Cells of the hypodermis and cortex divide in response

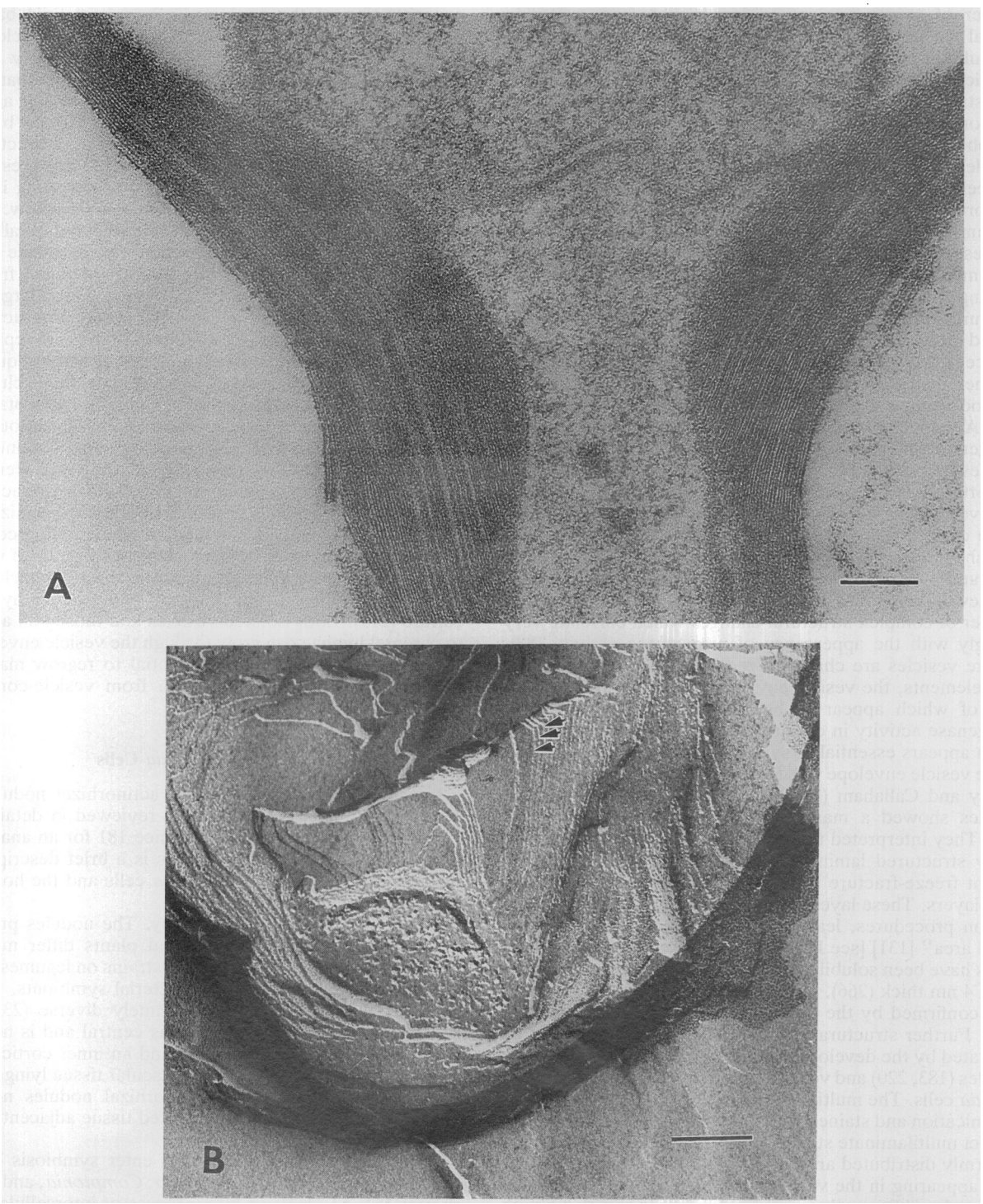

FIG. 4. Transmission electron photomicrographs of the *Frankia* envelope visualized by permanganate fixation (panel A) and freeze-fracture (panel B). (A) View through the stem region of a vesicle after preparation by sonication and permanganate fixation. Note the laminated layers corresponding to the laminae visualized by freeze fracture in panel B. (B) Appearance of the lipid monolayers on the surface of a vesicle induced under 40% O_2 and prepared by freeze fracture. Arrows indicate lipid laminae. Bars, 0.1 μm. Panel A reproduced from reference 104 with permission.

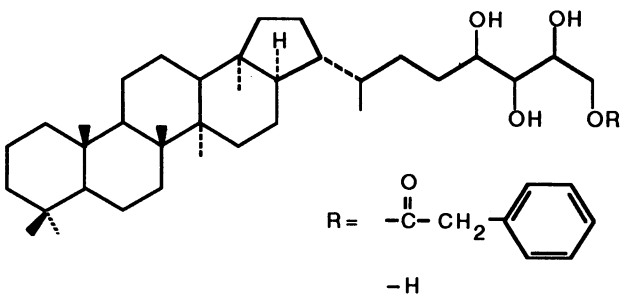

FIG. 5. Structure of bacteriohopanetetrol and its phenylacetate monoester as identified in reference 46.

to the invasion to form a prenodule (25), and the *Frankia* hyphae penetrate through the prenodule tissue into the inner cortex of the root. The nodule itself develops in the same manner as a lateral root, with primary nodule lobe primordia arising in the pericycle, endodermis, or cortex at the same time as prenodule development. The *Frankia* hyphae penetrate cells of the developing nodule lobe primordia to form the infected nodule.

Unlike most species, *Elaeagnus*, *Shepherdia*, and *Ceanothus* species are infected by intercellular penetration of the epidermis. In *Ceanothus* species this occurs in the presence of root hairs which are not infected, whereas in *Elaeagnus* and *Shepherdia* species infection occurs in the absence of root hairs. In this mode of infection, the *Frankia* hyphae have an extensive intercellular existence but are still encapsulated in a pectic capsule (147) which extends through the intercellular spaces. *Frankia* strains that infect both *Elaeagnus* and *Myrica* species (162) or both *Shepherdia* and *Gymnostoma* species (203) have been shown to enter either by intercellular penetration or by root hair infection depending on the host. Thus, the infection route is determined by the host plant, and the microorganism, if compatible, follows the appropriate route.

The infective hyphae of *Frankia* strains, as they enter the root hair, become encapsulated with a layer of plant cell wall-like material surrounded by host plasmalemma. This encapsulation is apparently continuous with the host cell walls and surrounds all infective stages both during invasion and in the mature nodule (Fig. 6) (124, 129, 130). The encapsulation was originally shown to be pectinaceous in *Alnus* species (130). More recently, the *Frankia* capsule has been studied by using immunogold localization and fluorescein-conjugated alginate and pectate probes (40, 147), which have demonstrated the presence of cellulose, hemicellulose (xylans), and pectins (Fig. 6A). It is concluded, at least for *Alnus* and *Casuarina* species, "that the capsule is essentially a thin internal tubular plant cell wall" (40). In *Ceanothus* species the capsule has a very high concentration of polygalacturonans and appears to be somewhat distinct from the cell wall in this respect (147).

The invasive phase of nodule development is characterized by hyphae which pass through both the middle lamella

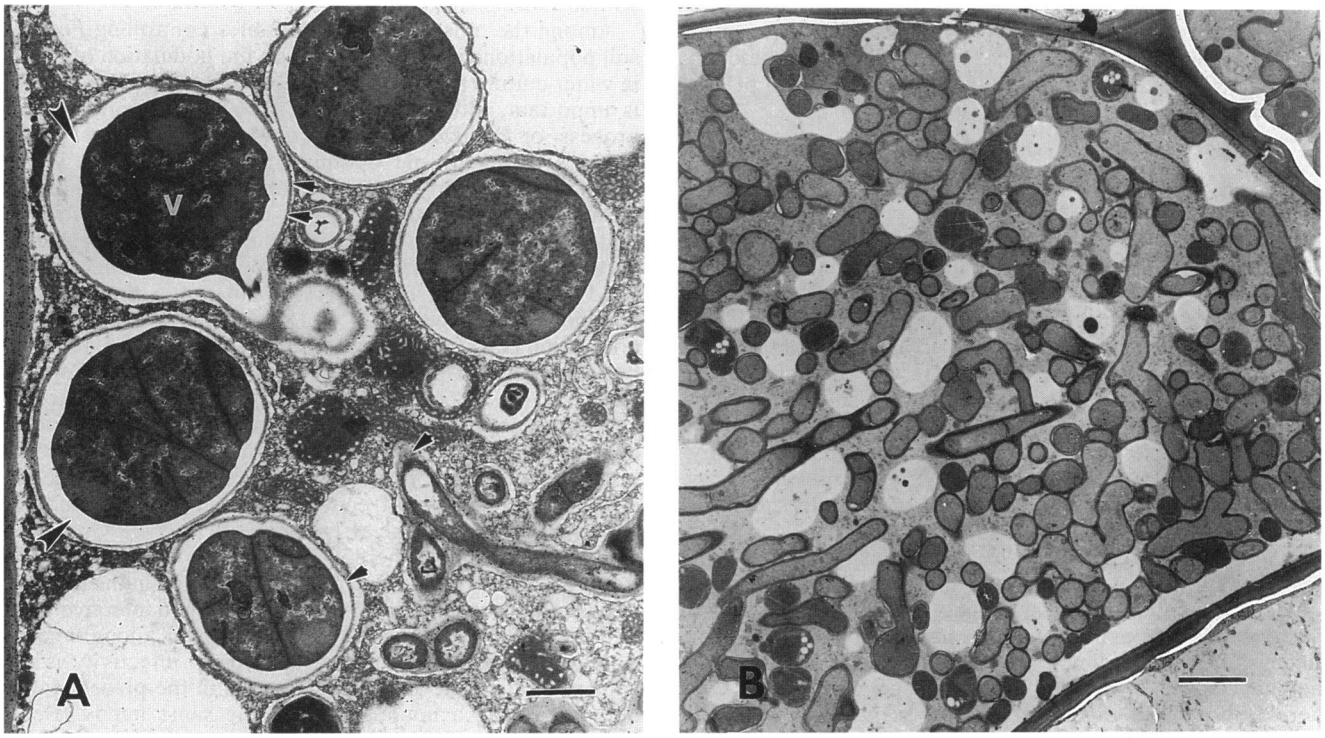

FIG. 6. Transmission electron photomicrographs illustrating the extremes of vesicle development in symbiosis. (A) Transmission electron micrograph of CF *Alnus rubra* nodules, showing fully developed vesicles (v) surrounded by a void area from which vesicle envelope material has been extracted (large arrows), and a plant cell wall-derived capsule (small arrows) that has retained colloidal gold-labeled cellulase used to localize cellulose polymers. (B) Transmission electron micrograph of FS *Casuarina cunninghamiana* nodules, showing the proliferation of *Frankia* hyphae without vesicle fomation. Bars, 2.0 μm. Photomicrographs courtesy of R. H. Berg.

of cell walls and the host cytoplasm. The hyphae differentiate into colonizing hyphae, which penetrate cell walls, and proliferating hyphae, which grow and branch in the center of host cells (181).

The mature effective form of *Frankia* strains in infected cells is often but not exclusively associated with symbiotic vesicle formation. At one end of the structural spectrum, the *Alnus* and *Elaeagnus* nodules have large, spherical, mulitseptate vesicles (Fig. 6A) and members of the Rosaceae (*Cercocarpus* and *Dryas* species) have nonseptate elliptical vesicles. At the other end of the spectrum, *Coriaria*, *Myrica*, and *Comptonia* species have simple club-shaped hyphal endings and *Casuarina* species have an entirely filamentous mature structure (Fig. 6B) (181). Since all effective *Frankia* strains described to date form vesicles in culture, it is apparent that the vesicle morphology in symbiosis is strongly controlled by the host plant. The first evidence that the plant controls the symbiotic form was obtained when it was found that *F. alni* HFPCpI1, which has club-shaped swellings in *Comptonia* species, had spherical vesicles in *Alnus* species and also in culture (126, 260).

Freeze fracture electron microscopy has resolved that the vesicles in symbiosis contain the multilayered envelope as they do in vitro (1, 131, 179). This work also demonstrated that the so-called void space, often shown around symbiotic vesicles, contains the lipid envelope, which, under standard procedures, is dissolved out by dehydrating agents. Evidence that the lipid envelope functions as an oxygen barrier in the symbiotic state was obtained by dark-field observation of *Alnus* vesicles in nodules of plants grown at various pO_2 values (234). However, when this work was repeated and vesicles were observed by freeze fracture techniques, there was little difference in the number of lipid lamellae other than at the tips of the vesicles (1). Given the difficulty of counting layers produced by freeze fracture electron microscopy and given the various ages of vesicles within a nodule, further study on the adaptability of *Frankia* cells to pO_2 in symbiosis is warranted.

ECOLOGICAL ASPECTS

Ecology in the Soil

Frankia strains occupy two distinct ecological niches, the root nodule and soil. Although a considerable amount of information is available on strains isolated from nodules and on the physiology of strains within nodules, the nature and extent of their free-living existence are still unclear.

The presence of soil-borne microorganisms capable of forming root nodules on plants other than legumes has been known since the last century (see reference 198 for a history of *Frankia* isolation). An early definition of *Frankia* species was subsequently made on the basis of cross-inoculations with crushed nodules. Total failure to obtain *Frankia* isolates from nodules led Becking (24) to the conclusion that *Frankia* strains were obligate symbionts and to establish *Frankia* species on the basis of host range. The isolation of viable *Frankia* strains from nodules (62) and from soil (17) and their successful culture in simple media lead to the conclusion that they have a free-living saprophytic existence in soil. As yet, there is no direct evidence for this view since isolation techniques do not differentiate between spores and hyphae; the evidence for the growth, persistence, proliferation, and survival of *Frankia* strains in soil comes from a range of indirect observations and experiments.

Many observations show *Frankia* strains to be present in soils well outside the normal geographic range of appropriate host plants (28) or long after host plants have disappeared from a site (210, 245, 279, 290). An example of this observation is the reliable nodulation of both *Alnus* and *Elaeagnus* species in New Zealand at every site where they grow from sea level to 1,700 m even though both genera are recent arrivals to the country. Likewise, *Casuarina* species, which originated in Australia, are nodulated when grown in Florida (272). In contrast, neither *Casuarina* (from Australia) nor *Ceanothus* (from North America) species are nodulated naturally in New Zealand (230a). Although some of these observations may reflect *Frankia* populations that persist in the absence of the appropriate host, they may also reflect the broad host range exhibited by some strains or the promiscuous nature of some of the host plants. For example, a study of 100 soil samples taken from different sites in Spain showed that all but 7 of the 84 soils capable of nodulating alder also nodulated *Myrica gale* despite the absence of *M. gale* from all but one of the sampling sites (44). It is now known that *Alnus* and *Myrica* species belong to the same host specificity group; moreover, *M. gale* is known to be a "promiscuous host," which enters into symbiosis with a broad range of *Frankia* strains.

Survival and persistence in soil have generally been studied by using host plants as bioassay agents for *Frankia* strains (243, 279). Using these techniques, it has been shown that the nodulation capacity of soil under *Alnus* species and under nonhost plants varied between 10^1 and 10^5 and between 0 and 3.1×10^3 nodulation units (nu) cm of soil^{-3}, respectively (245, 278, 279, 281). Using 20-fold soil dilutions, it is possible to detect 0.07 nu cm^{-3} (10 to 100 *Frankia* cells cm^{-3}) in soil (243). These techniques all confirm the widespread presence of *Frankia* strains in many soils with and without host plants (117, 243).

Among the most important variables controlling *Frankia* soil populations is the pH. Work on the nodulation of plants in water culture (86) and in soil (210, 246) has shown that pH is important, although whether pH affects the nodulation process, or *Frankia* survival, is not known. For most plants there is a narrow pH optimum near neutrality at which nodulation is optimal, and this coincides with the optimum growth pH of *Frankia* cells in culture. The number of nodulation units in soil, as measured by most-probable-number nodulation tests on *Alnus* species, is strongly correlated with soil pH up to pH 8.0 but not with any other common soil property (245). Despite these observations, nodulation can occur in soils with pH down to 3.5, possibly because of higher-pH microsites or acid resistance of spores (245).

The presence of host plants is obviously the major factor in maintaining and amplifying *Frankia* populations. In some instances, densities of *Frankia* cells in soil can be completely explained by release of endophyte from nodule turnover, assuming a survival time of 1 year in soil (9, 278). Growth in the host root rhizosphere has also been postulated (79). Removal of host plants or artificial inoculation in the absence of host plants usually results in a rapid drop in infective units to a low level which can be maintained over a long time. A significant exception to this steep drop in infectivity is the apparent amplification of *Frankia* cells in the presence of a *Betula* species, a nonhost tree in the same family (Betulaceae) as *Alnus* (243, 245, 279). Some very elegant scanning electron micrographs have shown luxuriant growth of *Frankia* cells on the roots of *Betula* and *Alnus* trees (244). It is significant that, in addition to hyphae, both sporangia and vesicles are formed in the absence of added carbohydrate,

suggesting that *Frankia* cells can both proliferate (sporulate) and presumably fix N_2, as well as grow outside root nodules in soil (244). These preliminary findings lead to the hypothesis that *Frankia* proliferation is primarily in the nodules of host plants and secondarily in the rhizosphere of both host and related species; in addition, some growth must occur in soils to sustain the numbers of *Frankia* cells that occur in the absence of host plants.

Although nodulation techniques are sensitive as bioassays for *Frankia* strains in soil, they are very time-consuming, do not discriminate between different strains, exclude noninfective strains, and neglect the effects of competition between strains (5). Recent attempts to identify *Frankia* strains in soil and in nodules by detection of specific DNA fragments after PCR amplification provide the opportunity for faster, more specific, and more direct analyses of *Frankia* ecology (177, 193). By using a labelled *nifHDK* probe and amplifying the *nifH* gene, *Frankia* strain ArI6 was detected down to 209 nu g of soil^{-1}, and, with modifications of the technique, it was suggested that a sensitivity of at least 2 nu g^{-1} could be achieved. rRNA has also been used for oligonucleotide detection and has proven to be useful in studies of nodules (101), but the sensitivity of the technique relative to PCR and problems with the quantification of results make it difficult to apply this technique to ecological studies with soil (5, 98). Recent work involving PCR of variable regions in *Frankia* rRNA genes has given estimates of 0.2×10^5 genomes of *Frankia* per g of soil in an established alder stand, which corresponds well to previous estimates based on bioassays (193).

Infectivity and Effectivity

Various ecological and functional groups of *Frankia* have been recognized over and above the normal taxonomic divisions and compatibility groupings. The groups "infective" (able to form nodules) and "effective" (capable of symbiotic N_2 fixation) are functional groups which have important ecological and physiological implications.

By far the majority of *Frankia* strains are infective. They have normally been isolated from nodules, and, until the advent of more sophisticated techniques, infection has been a major definition of the group. However, a number of strains which appear to be noninfective have been isolated (16, 100, 137). Identification of these isolates as authentic *Frankia* strains has generally been confirmed by morphological criteria and by chemical or genetic probes. A recent study has highlighted the problems inherent in dealing with one of the more recalcitrant genera, *Coriaria* (164). Nine isolates from *C. nepalensis* all failed to express nitrogenase in culture and did not form vesicles. The isolates were all confirmed as *Frankia* strains by hybridization with a 16S rRNA-targeted oligonucleotide probe and by cluster analysis of fatty acid profiles (165). Since the isolates came from the *Coriaria* nodules, the designation of the isolates as noninfective is obviously tentative and awaits more knowledge of the conditions under which nodules may be formed in genera such as *Coriaria*.

Much more is known about a range of infective but ineffective strains. They occur commonly in natural habitats (100, 137) and represent genetically distinct groupings of strains which are quite separate from effective types. Some strains may be ineffective on one host genus or species but effective on another (57, 115). Ineffective strains are generally incapable of forming vesicles in symbiosis (16, 282) and therefore lack the ability to fix N_2. Little is known of the competitive ability of the two groups if both are present in a given soil, but it seems likely that if high levels of ineffective strains occur, they would compete for infection sites with effective strains (282).

Host Specificity

Frankia strains exhibit various degrees of host specificity, but the rules for predicting specificity have proven something of a Gordian knot. As a starting point, Baker defined three or four host specificity groups (HSGs) among *Frankia* strains mainly on the basis of the results of cross-infectivity studies conducted in water culture on members of the Betulaceae, Myricaceae, Elaeagnaceae, and Casuarinaceae (22). HSG 1 is composed of strains that infect *Alnus* (Betulaceae) and *Comptonia* (Myricaceae) species; HSG 2 strains infect members of the Casuarinaceae (*Casuarina*, *Allocasuarina*, and *Gymnostoma* species); and HSG 3 strains infect members of the Elaeagnaceae (*Elaeagnus*, *Hippophaë*, and *Shepherdia* species). Members of HSG 1, HSG 2, and HSG 3 also nodulate promiscuous hosts from the genera *Myrica* (Myricaceae) and *Gymnostoma* (Casuarinaceae), which appear to be less selective than other plants in choosing a symbiotic partner. A fourth HSG defined by Baker (22) contained strains that nodulate members of the Elaeagnaceae but not the promiscuous hosts.

Although these groups are relatively straightforward in principle, the situation is complicated by the observation that a strain from one HSG can sometimes be isolated from a plant normally infected only by members of another HSG. Some of these isolates then fail to nodulate the source plant, or they nodulate the source plant ineffectively (16, 79, 100, 164). Such strains have been referred to as atypical. In addition, recent studies have demonstrated the existence of strains that have been isolated from *Elaeagnus* species and that form ineffective nodules on *Alnus* species (57). In the latter case, the strains isolated from the *Elaeagnus* species can also be considered flexible in the sense that infection is achieved via root hair penetration in *Alnus* species and by intercellular penetration in *Elaeagnus* species (162).

In most cases, isolates from *Alnus* species or members of the Elaeagnaceae generally will effectively nodulate their source plant. However, less predictability is evident among strains isolated from members of the Casuarinaceae or Myricaceae and recently isolated strains from members of the Coriariaceae or Rhamnaceae (64, 65, 165). In a study of 22 isolates from members of the Casuarinaceae, 9 failed to nodulate their original hosts and 2 additional strains nodulated less than half of the source host seedlings tested (267). Four of the noninfective strains were tested on *Elaeagnus* seedlings, and all produced nodules. Of these four strains, two (D11 and CcI2) have since been included in 16S rRNA analyses and DNA-DNA homology studies (87, 178). Strain D11 clustered with strains isolated from *Elaeagnus* species by rDNA analysis and had no detectable DNA homology with other strains from *Casuarina* species (genomic species 9); CcI2 clustered with typical strains isolated from *Casuarina* species belonging to genomic species 9 by rDNA analysis and had 38% DNA homology with genomic species 9. Thus, considerable diversity can be found among atypical isolates.

The simplest explanation for the common isolation of atypical *Frankia* strains is that atypical strains infect primarily other actinorhizal plants and induce root nodules on secondary hosts when certain environmental conditions are met. The promiscuous hosts, *Myrica* and *Gymnostoma*

species, which are infected by members of HSG 1, HSG 2, and HSG 3 (at least) and thus yield a variety of strains, may simply represent the extreme case along a spectrum of competence. As mentioned above, some strains isolated from *Elaeagnus* species can ineffectively nodulate *Alnus* species at low frequency (57), and some strains isolated from *Alnus* species reportedly nodulate *Coriaria* species (211); therefore, finding such strains in field nodules should not be surprising. Other explanations advanced to explain this phenomenon include (i) the presence of more than one strain in a nodule and (ii) loss of the genetic information for plant nodulation on passage through culture. More than one strain has been isolated from a single nodule in the past (37, 81); therefore dual infection is possible, and adequate care should be taken to obtain monocultures. However, genotypic changes of the magnitude required to convert an alder- or casuarina-isolated strain to an elaeagnus-isolated strain seem unlikely. Finally, the existence of promiscuous *Frankia* strains, which have few scruples in their choice of partner, has not yet been ruled out.

The host specificity picture will no doubt become more complex as additional strains are isolated from other members of well-studied plant families and from plant families that have yet to yield isolates that reinfect their source plant species (members of the Coriariaceae, Datiscaceae, Rhamnaceae, and Rosaceae). Practical concerns that must be addressed include the effect of plant cultivation methods on apparent competence, the incidence of multiple strains within a nodule, and the effect of intra- and interspecies plant differences that may determine susceptibility. In this regard, some studies have shown that different plant species within a genus or different ecotypes or clones of the same host plant can respond differently to nodulation by a given *Frankia* isolate (57, 100, 112, 238). In a general sense, the relatively broad spectrum of plants infected by individual *Frankia* strains implies that the plant-bacterium interactions needed to establish a symbiosis may be less complex, or at least more general, than those in the rhizobium-legume symbiosis.

CULTIVATION OF FREE-LIVING *FRANKIA* STRAINS

Isolation of *Frankia* strains

When designing protocols for isolating *Frankia* strains, the two major factors limiting success are the slow growth of *Frankia* strains and the presence of contaminants. Little can be done to improve the growth rate, but it is possible to minimize and even eliminate nodule surface contamination. A number of surface disinfectants have been used for this purpose, most commonly dilute sodium hypochlorite and osmium tetroxide (142). In some methods, washed nodule pieces can be embedded in solid media or incubated in liquid media for prolonged periods (128). A drawback to this approach is that the clonal nature of the subsequent cultures is not ensured. A better approach used in some techniques is to fragment the surface-sterilized nodule lobes and plate dilutions of the resulting vesicle cluster suspensions in or on solid media or dilute to extinction in liquid media (31, 79). Vesicle clusters consist of a hyphal mass plus vesicles and plant cell cytoplasmic debris, and sometimes *Frankia* sporangia, that occupy a single infected plant cell in symbiosis. The advantages of using a dilution plating technique are that the outgrowth of hyphae from vesicle clusters can be monitored microscopically, with the chances of obtaining a monoculture improved accordingly, and any contaminants that arise are spatially removed from the slower-growing *Frankia* colony. Approaches involving differential filtration (31, 55) or sucrose density gradient centrifugation (17, 21) to concentrate and wash vesicle clusters appear to be the most successful (142).

The medium used in isolating new *Frankia* strains is critical for success, but no universal or selective medium has yet been devised. Several general media for isolation and growth have been reported (142). Those that have proven effective range from simple tap water agar to defined propionate media (17) to the complex Q Mod medium of Lalonde and Calvert (127). Richer media tend to favor the growth of contaminants. Antifungal agents, such as cycloheximide or nystatin, are often incorporated to minimize fungal contamination. Some strains require the addition of a root-lipid extract to stimulate their initial growth during isolation (199). The active substance in the root extracts has been identified as the steroid hydroxydammarenone II (dipterocarpol) (200). Such a requirement has so far been reported in relatively few cases. However, the inability to isolate *Frankia* strains from some plant families may reflect special requirements that have not yet been identified. Furthermore, structural effects or toxic properties of some types of nodules may preempt isolation. For example, it has been observed that, although some strains that are readily reisolated from alder nodules can infect *Coriaria* species, they could not be reisolated from *Coriaria* nodules (211). In general, it is best to use a range of media in initial isolation trials, particularly since the emergence of identifiable *Frankia* colonies may take from 10 days to 1 year after plating (142). Once *Frankia* strains have been isolated, it is essential to ensure that cocultures have not been obtained. Usually, this is accomplished by the subculturing of single-spore isolates through several rounds of plating and growth.

Growth Characteristics

All *Frankia* strains isolated to date are obligate heterotrophic aerobes that grow slowly, with doubling times of 15 h or more. Some strains prefer lower O_2 levels than others and grow poorly in vigorously aerated media, particularly if a sparse inoculum has been used. As a consequence of their filamentous growth habit and the often abundant sporangia formed in liquid culture, growth kinetics of *Frankia* strains are complex, generally consisting of a stationary phase after homogenization and transfer, followed by a short exponential phase and then by a slower increase in biomass over time. This pattern of growth varies with the inoculum density, medium, and degree of agitation. The usual problems in dealing with a mycelial organism apply to *Frankia* strains, including the development of nutrient and waste gradients through mycelia, the absence of true balanced growth, and a tendency to form flocs during incubation (reviewed in reference 38). An understanding of these problems has led to improved short-term cultivation methods that yield consistent results (105, 169, 174, 183, 218, 220, 221, 292). In addition, progress has been made in continuously cultivating N_2-fixing *Frankia* cells by using a semicontinuous approach (105).

Growth and maintenance media range from simple basal-salts media containing a suitable carbon source to complex media containing, in addition to basal salts, yeast extract, bovine serum albumin, malt extract, beef extract, NZ Amine A, Casamino Acids, vitamin supplements, or Tweens (142). Virtually all *Frankia* strains isolated to date are prototrophs that require no identified growth factors and therefore grow well in minimal media. Some strains are inhibited by yeast

extract or other undefined media additives (142). The best approximation of a general medium for *Frankia* is the DPM (defined propionate minimal) medium of Baker and O'Keefe (17).

Most *Frankia* strains are grown and maintained in liquid culture and generally grow slowly on solid media, which are used mainly for purifying cultures, for assessing spore germination, or for identifying mutants (196, 273). Colonies from spores or mycelial fragments become visible to the unaided eye about 7 to 10 days, or longer, after plating, under the best of conditions (143a). In our experience, some types of agar are inhibitory, whereas gellan gum, a solidifying agent derived from *Pseudomonas elodea* capsular polysaccharide, has proven to be a suitable substitute, especially for spore germination studies.

PHYSIOLOGY OF FREE-LIVING *FRANKIA* STRAINS

Carbon Metabolism

In accordance with their genetic diversity, *Frankia* strains differ in the range of substrates they use for growth. *F. alni* (genomic species 1) strains are the best defined. They generally grow well on short-chain fatty acids (acetate, propionate); variably on succinate or malate, Tween 80, and pyruvate; and poorly, if at all, on a range of sugars tested. This narrow range contrasts with some strains from genomic species that infect the members of the Elaeagnaceae and Casuarinaceae; these strains grow on sugars as well as organic acids (38). The original physiological groups proposed by Lechevalier and Lechevalier—groups A and B—are still largely intact (140). Thus, *F. alni* strains constitute physiological group B, and most strains that infect members of the Elaeagnaceae or Casuarinaceae generally belong to group A. Since most of the carbon source utilization experiments were done soon after *Frankia* strains became available but before genomic groups were established, much work remains to be done on determining the degree to which simple physiological traits correlate with more recently established genomic species.

General pathways of carbon metabolism have been described for a few *Frankia* strains. Individual enzymes involved in the Embden-Meyerhof-Parnas glycolytic pathway, pentose phosphate pathway, tricarboxylic acid cycle, glyoxylate cycle, and gluconeogenesis have been detected primarily in *F. alni* AvcI1 and HFPArI3 (genomic species 1) and in HFPCcI3 (genomic species 9) (53, 151, 250). From ^{14}C-labeling experiments, propionate assimilation and metabolism have been proposed to proceed via active transport followed by activation to propionyl coenzyme A, biotin-dependent carboxylation to methyl-malonyl coenzyme A, and then racemization to succinyl coenzyme A, which enters the tricarboxylic acid cycle pools (250).

Glycogen and trehalose have been identified as major storage compounds in some *Frankia* strains (150), with glycogen deposits visible in transmission electron micrographs of cultured and most symbiotic *Frankia* strains except for frankiae in *Discaria* and *Dryas* nodules (181). Vesicles that are actively involved in N_2 fixation generally appear to have fewer glycogen granules than do young developing vesicles or older vesicles no longer involved in fixing N_2 in symbiosis (36, 88). These observations most probably reflect the high-energy requirement for N_2 fixation in vesicles, where much of the incoming substrate would be needed for energy generation rather than storage compound synthesis. The availability of techniques for isolating vesicles has opened the possibility of studying vesicle carbon metabolism in both culture and symbiosis (183, 220), although only one study to date has taken advantage of this approach for studies on symbiotic frankiae (284).

Extracellular enzymes. *Frankia* strains have been reported to produce extracellular cellulase, pectinase, and proteinase activity in pure culture (169, 215, 224). Cellulases have been detected in strains HFPCcI3, HFPCcI2, and HFPGpI1, which are derived from members of the Casuarinaceae, and in *F. alni* HFPCpI1 and HFPArI3, but no growth on cellulose was observed (215). Of these strains, HFPCpI1 exhibited the highest carboxymethyl cellulose-hydrolyzing activity and HFPCcI3 completely degraded filter paper strips. Given the fact that HFPCpI1 and other *F. alni* strains do not grow, or grow poorly, on simple sugars, the presence of cellulase in these strains may suggest that appropriate conditions for growth on sugars have not been identified or that the cellulase has been maintained in some *Frankia* strains for plant infection or other purposes.

Low levels of pectinase activity have been detected by a cup-plate pectin agar assay in *F. alni* ACN1^{AG} and HFPCpI1, and possible *pel* genes have been detected by Southern blotting with the *Erwinia chrysanthemi pelBDA* genes as a probe in ACN1^{AG} and other strains. Proteolytic enzymes have been identified in culture supernatants of *Frankia* strain BR (genomic species 9 of the strains infecting members of the Casuarinaceae [87]), some in the form of high-molecular-mass proteosomes (29, 169).

The various hydrolytic enzymes in culture supernatants may play some roles in the infection process, or in the maintenance of *Frankia* strains in symbiosis. Cellulose, cellulose microfibrils, xylans, and pectins have been found associated with the plant-derived capsules surrounding frankiae in several actinorhizal plants (1, 40, 130, 146, 147, 181); the capsule has been likened to an intracellular, and tubular, primary plant cell wall (40), and capsular polymers have been proposed to serve as sources of energy in symbiosis (40, 130, 251), although no studies have been done on the kinetics of deposition versus degradation of capsular material. In addition, the amino acid sequences cleaved by extracellular proteinases produced by *Frankia* strain BR resemble extensins found in plant cell walls; on the basis of this specificity, a role in the infection process was proposed (29). Thus, the hypothesis that *Frankia* strains degrade plant cell wall polymers during their traversal of cell walls and during fixation of N_2 in mature infected cells is attractive but unproven at present.

Nitrogen Metabolism

Frankia strains can use a variety of organic and inorganic sources of nitrogen for growth, including amino acids, urea, nitrate, ammonia, and N_2. Surveys of such compounds have not been extensive, so the variability of N source utilization among genomic species is not clear. Like studies on C metabolism, most information concerning the physiology of N_2 fixation, ammonia assimilation, and amino acid metabolism has been obtained from studies on strains of *F. alni* and the *Casuarina*-infecting strain HFPCcI3.

Nitrogenase and O_2 protection. In accord with the highly conserved nature of nitrogenase and associated proteins in prokaryotes, *Frankia* nitrogenase is O_2 labile, requires $Mg \cdot ATP$ and reducing power, and produces NH_4^+ and H_2 gas in an ATP-dependent fashion (32). Nucleotide and amino acid sequence analyses of *nifH*, *nifD*, and other *nif* genes confirm the similarity of *Frankia* nitrogenase with the clas-

sical MoFe-protein-based systems (103, 184, 190, 239). No alternative N_2-fixing systems akin to the vanadium- or iron-based nitrogenases have yet been reported from frankiae. Since frankiae grow and respire slowly, the delivery of substrates to nitrogenase and the maintenance of a low O_2 level in the proximity of nitrogenase are important problems encountered by *Frankia* strains.

A characteristic of nearly all *Frankia* strains tested, and in strict contrast to *Rhizobium* strains, is the ability of aerobically grown cells to fix N_2 (93, 259, 260). A number of studies, both in symbiosis (116, 160, 216) and in culture (158, 159, 174, 183, 253, 254, 260), demonstrate that nitrogenase activity is correlated with the presence of vesicles and that nitrogenase is localized within the vesicles. These studies involved a variety of approaches, including direct assay of nitrogenase in isolated vesicles (183, 254), immunolocalization (116, 157, 159, 216), and correlative work on vesicle development and nitrogenase induction (174, 253, 260). However, at very low pO_2 (0.1 to 0.3 kPa in the atmosphere), HFPCcI3 cells are able to express nitrogenase in the absence of vesicles (176). The work of Murry et al. (173) and Parsons et al. (192) has led to the conclusion that the vesicles provide the diffusion resistance to oxygen that is required to maintain a low internal pO_2. Thus, under normal conditions, nitrogenase is localized in vesicles, where it is protected by an oxygen-limiting external lipid envelope. Under extreme O_2 limitation in culture or in symbiosis, vesicles are not formed and nitrogenase is found in the hyphae.

A unique feature of *Frankia* vesicles is their ability to adapt to various ambient pO_2. *Frankia* cells grown at pO_2 levels from 2 to 70 kPa adapt to those levels and show optimum nitrogenase activity at or near the pO_2 level at which they were grown (192). Torrey and Callaham (266) were the first to identify the laminated wall structure of the vesicle and to postulate that this may be the site of O_2 protection in *Frankia* cells. Vesicles show distinctive changes in their brightness under dark-field microscopy, with low-pO_2 vesicles appearing very thin and those growing under high pO_2 appearing thick walled and bright (106, 192). These microscopic observations correlate well with the number of lipid layers in the vesicle envelope, varying from 17 at 4 kPa of O_2 to an average of 40 at 40 kPa of O_2 (192). There seems little doubt that the physiology of oxygen protection in *Frankia* cells is determined partly by envelope thickness, and it has been confirmed in steady-state growth studies that the vesicle envelope thickness is maintained by, and is very responsive to, ambient pO_2 (106).

The abundance of hopanoids in the *Frankia* vesicle envelopes (see above) and their role in membrane stabilization provide forceful arguments for their involvement in limiting O_2 diffusion to the vesicle interior, in a manner similar to that proposed for glycolipids in cyanobacterial heterocyst envelopes (107). Thus, a combination of respiration for internal removal of O_2 coupled with a diffusion barrier across the vesicle envelope may be sufficient to provide an anaerobic environment for nitrogenase to function.

It is important to note that thickening of the vesicle envelope by laying down extra lipid layers is a developmental process that is specifically under oxygen control and is essential to nitrogenase functioning. The induction of vesicles is independent of ambient pO_2, above a certain level, but the induction of nitrogenase activity within vesicles is entirely coincident with the envelope thickening (106). Thus, HFPCcI3 vesicles induced under 5 kPa of O_2 showed nitrogenase activity 91 h after induction, whereas those grown at 40 kPa of O_2 did not display nitrogenase until 170 h after induction (106).

Ammonia assimilation and amino acid metabolism. Ammonia is assimilated by free-living *F. alni* HFPCpI1 via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, leading to the production of glutamate and glutamine (38). Alanine dehydrogenase and glutamate dehydrogenase have been detected but are probably not involved in primary ammonia assimilation since their levels remain low and unresponsive to changes in ammonia levels (220). This conclusion is supported by studies involving [^{13}N]ammonia labeling of nitrogen-starved HFPArI3 cells. Label accumulated primarily in glutamine, with much lower labeling of glutamate, alanine, and serine (50).

When the cells are growing on high concentrations of ammonia (>0.5 mM), ammonia apparently enters the cells via passive diffusion of the unprotonated form (156). At low concentrations of ammonia, or in combined-nitrogen-free media, a specific ammonium permease is induced, allowing HFPCpI1 to scavenge ammonium down to undetectable levels by an active-transport mechanism (156). On entry into nitrogen-starved cells, ammonia (or its transport analog methylammonium) is rapidly assimilated by GS to glutamine (or γ-*N*-methylglutamine) (156). Given the high affinity of the transport system and the ability of GS to rapidly assimilate ammonia in free-living *Frankia* cells, a substantial modification of these systems must occur during the transition to the symbiotic state in which ammonia is thought to be released by the vesicles.

Studies of the regulation of GS in *Frankia* strains are complicated by the presence of at least two GSs, designated GSI and GSII. The former is biochemically and genetically similar to classical prokaryotic GSs; it is a dodecamer of identical subunits, regulated by adenylylation-deadenylylation and is produced at relatively constant levels in all growth media tested (84, 269, 292). The latter is an octamer of identical subunits, is not adenylylated, and is similar in structure and amino acid sequence to eukaryotic GSs (209); it is produced in response to growth on N_2, glutamate, or amino acids that are degraded to glutamate, such as histidine, aspartate, and proline (269, 292). Essentially the same conditions that lead to an induction of vesicles and nitrogenase are sufficient for GSII induction. Nitrogen sources catabolized as ammonia or glutamine, such as glutamine, do not lead to GSII synthesis or to vesicle differentiation. The behavior of GSI, GSII, and nitrogenase in free-living *Frankia* cells resembles the behavior of analogous nitrogen-regulated systems in enteric bacteria and rhizobia and argues for the presence of similar regulatory mechanisms in *Frankia* cells.

In culture, GSII is found in both vesicles and hyphae (220, 269, 292). The relationship of the dual-GS system to N_2 fixation and symbiosis is probably not direct, since other actinomycetes (27, 123) and members of the family *Rhizobiaceae* (71) have physiologically and genetically similar multiple GSs.

Beyond the initial steps of ammonia assimilation, few published papers have addressed amino acid metabolism in *Frankia* strains. In an early study of *F. alni* AvcI1, GS and glutamate oxaloacetate transaminase (GOT) were detected but GOGAT, glutamate dehydrogenase, alanine dehydrogenase, aspartate dehydrogenase, tyrosine, leucine and alanine aminotransferases, aspartase, acetylornithine glutamate transacetylase, and L-amino acid oxidase were not detected (8). *F. alni* HFPCpI1 has GOGAT, alanine dehydrogenase, and glutamate dehydrogenase activities as well as GS and

GOT (220). In young cultures of HFPCpI1 transferred to fresh medium containing [^{14}C]aspartate, label was rapidly accumulated in glutamate followed by glutamine/asparagine (292). In older cultures, label accumulated mainly in aspartate and more slowly as glutamate. Thus, aspartate is most probably catabolized by GOT to oxaloacetate, which enters the tricarboxylic acid cycle, to α-ketoglutarate and then to glutamate via GOGAT. The rapid filling of glutamate pools appears to be a priority for newly transferred cells; this is not surprising given the central role of glutamate in amino acid metabolism in bacteria, including *Frankia* strains (50). Aspartase, which would convert aspartate to ammonia and fumarate and presumably would repress vesicle formation, was not detected in HFPCpI1 (292). Interestingly, cells growing on 10 mM aspartate formed vesicles and had about threefold higher nitrogenase activity than did cells growing on N_2 or on 1 mM aspartate. This stimulation may be significant in symbiosis, during which *Frankia* cells are exposed to amino acid pools of the plant. Aspartate has recently been suggested to be an essential substrate for rhizobial bacteroids, since aspartate aminotransferase mutants fail to form an effective symbiosis (205).

Vesicles isolated from N_2-grown cultures of HFPCpI1 contain, in addition to nitrogenase, both forms of glutamine synthetase (GSI and GSII) and GOT but not GOGAT (218, 219, 292). The presence of GS activity and the absence of GOGAT activity suggest that vesicles may be capable of forming glutamine from ammonia and glutamate but may not be capable of converting glutamine to glutamate. If this is correct, *Frankia* vesicles may be physiologically analogous to cyanobacterial heterocysts, which fix N_2 and transport glutamine to adjacent vegetative cells (107). Also, this model implies that GS syntheses in vesicles and hyphae are regulated differently, such that GSII synthesis would continue in the presence of glutamine in the vesicles.

Hydrogenase. It has been known for many years that actinorhizal nodules, with a few exceptions (212, 225), emit little nitrogenase-generated H_2 gas (167, 217). This observation contrasts with the highly variable production of H_2 by rhizobium-induced root nodules on legumes (217). Uptake hydrogenases have been demonstrated in symbiotic *Frankia* cells (33, 163, 212), and in free-living *F. alni* strains (145, 175). In both cases, electrons from hydrogen oxidation are eventually donated to O_2 (33, 175). Thus, the possibility exists that hydrogenase can help protect nitrogenase from O_2 inactivation through the oxyhydrogen reaction and can help regenerate ATP by recycling electrons through electron transport reactions. Evidence for these roles has been provided by studies involving the incubation of N_2-fixing cultures in the presence of unusually high levels of O_2 plus H_2 (175). Under these conditions, enhanced nitrogenase activity was detected, suggesting that the presence of a hydrogenase system ameliorated the destructive effects of O_2 or recycled electrons to provide additional energy. Immunogold localization studies suggest that hydrogenase is present in vesicles and hyphae of N_2-grown cells and in the hyphae of ammonia-grown cells, when cultures are incubated in an H_2-containing atmosphere for 16 h prior to harvest (145). In the only symbiotic system studied, *Alnus incana*, immunogold labeling identified hydrogenase in both hyphae and vesicles, although no hydrogenase activity could be detected (226). An interesting question is whether hydrogenase is present in vesicles or hyphae that were not exposed to H_2 before harvest. If hydrogenase is indeed inducible in ammonia-grown cells, *Frankia* strains, like some rhizobia (102), may be able to grow autotrophically.

METABOLISM IN SYMBIOSIS

Carbon Metabolism

In symbiosis, vesicle cluster suspensions from *Alnus* species respire when provided with NADH (7), succinate (8), phosphorylated hexoses plus pyridine nucleotides in various combinations (284), trehalose and other sugars (152), or a combination of malate, glutamate, and NAD^+ (6, 118). A malate-aspartate shuttle was proposed as a possible explanation for the observed respiration on malate, glutamate, and NAD^+, since malate dehydrogenase and glutamate-oxaloacetate aminotransferase were also detected in vesicle clusters. Providing evidence for the existence of such a system is problematical when using symbiotic *Frankia* strains, since vesicle clusters appear to be permeable to many low-molecular-weight molecules including phosphorylated hexoses, NAD^+, and ATP (284). This permeability may reflect intrinsic membrane instability in symbiotic *Frankia* strains or a difference between symbiotic hyphal and vesicle cell types, or it may be a consequence of the physical disruption of nodule tissue. In addition, the presence of plant organelles embedded in vesicle clusters presents a problem for respiration studies since plant mitochondria and mitochondrial membranes can participate in O_2 uptake reactions with some of the substrates provided (8). Thus, despite improvements in methods of vesicle cluster isolation (285), physiological studies on vesicle clusters are still subject to difficulties encountered in preparing material. At present, the carbon substrates donated by the plant to the microorganism remain unknown. Studies with well-defined mutants would greatly accelerate progress in this area.

Nitrogen Metabolism

To date, vesicle clusters that are capable of fixing N_2 on their own or when supplied with an oxidizable carbon source have not been obtained, although nitrogenase can be assayed directly in anaerobically prepared vesicle clusters by using sodium dithionite as a reductant and added Mg^{2+} and ATP (35, 283). Since nitrogenase remains intact, the ability of isolated vesicle clusters to generate reducing power and ATP must be greatly diminished from the symbiotic state and may reflect a general disruption of ion gradients and other cellular components.

As in free-living cultures, nitrogenase appears to be limited to vesicles in symbiosis, as judged initially from studies on *Alnus* nodules, in which the onset of nitrogenase activity correlates with vesicle appearance (160) and the loss of nitrogenase activity in winter coincides with nodule and vesicle degeneration (288). More direct evidence by way of immunolocalization studies on *Alnus* and *Elaeagnus* nodules (116, 216) confirm that the vesicle is the main site of nitrogenase proteins. In actinorhizal plants in which the vesicle is club shaped and vesicle envelopes are less developed, as in *Myrica* or *Comptonia* species, or those in which obvious vesicles are absent, as in nodules of *Casuarina* and *Allocasuarina* species, or in *Coriaria* nodules, in which filamentous symbiotic vesicles are evident, the location of nitrogenase is only speculative at present.

Ammonia-assimilatory enzymes of *Frankia* cells in symbiosis appear to be regulated differently from those in free-living cells. Studies with polyclonal antibodies raised against *Rhodospirillum rubrum* GSI and *Rhizobium meliloti* GSII indicate that the analogous *Frankia* GSs in symbiotic HFPCpI1 are absent or are present in very low abundance (154) and support previous reports of low GS activity of

vesicle clusters in symbiosis (54). The probable result of low levels of GS is that fixed nitrogen, in the form of NH_4^+, remains unassimilated in the bacterium and is rendered available to the plant either through simple diffusion of NH_3 or by another unidentified mechanism. The symbiotic absence, or lowering, of GS activity is a theme repeated in certain cyanobacterial (43, 120, 144) and rhizobial (228) symbioses, so that the *Frankia* symbiosis is not unusual in this respect, although the convergence of physiological strategies in N_2-fixing symbioses is remarkable.

Oxygen Protection

Evidence from structural and physiological studies indicates that a variety of mechanisms for limiting O_2 diffusion to nitrogenase exist in symbiosis. It is informative to compare the structure of legume nodules with that of actinorhizas. Whereas the legume nodule has a consistent structure in which the infected zone lies within a diffusion-resistant barrier, several structural motifs are evident in actinorhizal nodules (233, 263). Most nodules have large numbers of air spaces, and the stele is present inside the infected zone, suggesting that there is only limited resistance to gas diffusion to the outer surface of infected cells. Microelectrode studies (255) and several studies involving India ink penetration of gas-evacuated actinorhizal nodules (232, 255, 262) all indicate that air can penetrate readily to the surfaces of infected cells or at least to the outer sides of clumps of infected cells in *Casuarina* species (291).

Two other elements in the oxygen protection network of nodules, vesicles and hemoglobin, also show great variation in actinorhizal nodules, but they vary inconsistently. At one end of the spectrum of variation, *Casuarina* and *Myrica* nodules do not contain spherical vesicles but do contain hemoglobin (233, 256, 257), and this is consistent with a low measured pO_2 within the infected-cell areas, where one may expect to find hemoglobin to act as an oxygen transporter (255). At the other end of the spectrum, *Alnus* nodules have very well-formed vesicles and a much lower level of CO-reactive heme compounds (256). Although there is some evidence that vesicles in *Alnus* species may play a central role in oxygen protection (234), the evidence from electron-microscopic work on the vesicle envelope shows only marginal changes in the number of lipid layers in *Alnus* nodules grown at different pO_2 (1). The picture is confused somewhat by *Coriaria* nodules, which have very ill-defined vesicles, lack hemoglobin, are very well aerated, but show much of the physiology of legume nodules (232).

There is a strong temptation to search for a single unifying mechanism of O_2 protection in the actinorhizal nodules. However, the enormous diversity of host plants and of *Frankia* structure within nodules has resulted in a very variable physiology. There is evidence that the vesicle (234), the host cytoplasm (291), modified cell walls of infected cells (39, 42), and some components of the nodule (258) may act singly or in concert in providing the delicate oxygen balance required in actinorhizal nodules.

Actinorhizal nodules generally show an optimum nitrogenase activity at around 20 kPa of O_2, with significant inhibition above 25 kPa of O_2 (214, 234, 235, 258, 289). In most cases studied, there is no apparent adaptation to pO_2 levels above ambient. The exceptions are *Coriaria arborea* nodules, which have many of the responses displayed by legume nodules (232). Nitrogenase activity appears to adapt at O_2 concentrations above ambient levels, and nodules grown at 5 kPa of O_2 have rates of nitrogenase activity similar to rates at 30 kPa of O_2 (232). It thus appears that some actinorhizal nodules can change the resistance of some component of the diffusion barrier to accommodate a wide range of pO_2.

Actinorhizal nodules show a very rapid switch-off and recovery of nitrogenase activity in response to small increments in O_2 in the gas stream (214, 232, 234, 235), which have been called oxygen transients (236). The effect is also seen in free-living *Frankia* cells and is therefore not a nodule effect (236). An interpretation of this effect is that a rapid change in pO_2 within the nodule, or in *Frankia* vesicles in culture, results in an instantaneous switch-off of nitrogenase (conformational protection). The oxygen is then removed by respiration, which allows the nodule or vesicle to return to normal activity. Alternatively, the oxygen transient may reflect a temporary shift of electron allocation from nitrogenase to O_2 as the electron acceptor becomes available. The fact that these transients are such a feature of actinorhizal nodules and are so insignificant in legume nodules (113) lends further support to the hypothesis that any oxygen protection mechanism is close to the site of nitrogenase in actinorhizal nodules.

GENETICS

Introduction

Reviews of *Frankia* genetics (170, 189, 241) are available, so only a brief overview will be given here. At present, it is clear that this area of *Frankia* biology is brimming with the opportunity for the study of plant-microorganism interactions and is ripe for exploration by new and innovative approaches. Studies of *Frankia* genetics have been difficult for a variety of reasons, including relatively low growth rates, poor spore germination in most strains, lack of useful vectors, and the difficulty in attracting funding for projects that have to begin with the development of a new set of genetic tools. To date, phages, conjugative plasmids, transposable elements, R-plasmids, and standardized mutagenesis protocols have not been identified. Some progress has been made, but the genetic manipulation of *Frankia* strains is presently far from routine.

Many of the approaches used to date, and questions asked, have their intellectual roots in *Streptomyces* and rhizobial genetics, which flourished during the 1980s. Thus, attempts have been made to use *Streptomyces* plasmids and phages as vectors, with no success to date (186, 241); protoplast technologies have been developed but have not yielded successful fusion products either between *Frankia* strains or between *Frankia* and *Streptomyces* strains (reviewed in reference 241). One exception is the work of Prakash and Cummings (195), who reported protoplast fusion of the *Alnus rubra*-isolated *Frankia* strain NPI0136010 and *Streptomyces griseofuscus*. The fusants had a number of interesting chimeric properties; one produced N_2-fixing root nodules without vesicles. Unfortunately, the strains obtained have been subsequently lost (NPI, personal communication).

A few studies have reported the presence of sequences that hybridized to or complemented a mutation in the common *nod* genes associated with the infection process in rhizobia (67, 83, 207). However, studies of *nod* hybridizable DNA and functional complementation of *nodA*::Tn*5* mutants of *Rhizobium meliloti* have not proven fruitful after further characterization (206). A more recent report of *nodD*::Tn*5*-complementing activity in *R. leguminosarum* by

a plasmid containing *Frankia* DNA (67) must be confirmed by sequence analysis of the putative frankial *nodD* and by the demonstration of Tn*5* in the rhizobial *nodD*.

Several groups have attempted to use indigenous *Frankia* plasmids as transformation vectors either intact or modified as shuttle vectors with *Escherichia coli* replicons, particularly using an 8.3-kbp HFPCpI1 (ArI3) plasmid designated pFQ31 (68, 69, 241, 247). Marginal success has been achieved in introducing DNA and other macromolecules into *Frankia* cells, but constraints imposed by the low grow rate of strains and difficulties in demonstrating plasmid maintenance have slowed progress in this area (68, 69). The most fruitful areas of study have involved genome description and the cloning and sequencing of genes obtainable through heterologous hybridization.

Chromosome Composition

The genome molecular weights of *Frankia* strains ArI4 and EuI1 were determined to be 8.3×10^9 and 6.0×10^9, respectively, by reassociation kinetics analysis (10). These molecular weights correspond to genome sizes of about 12,000 to 8,700 kbp, similar to the reported genome size of *Streptomyces* spp. (58) and about twice as large as the genome of *E. coli* (4,700 kbp) (153). The DNA is 66 to 75 mol% G+C (11, 87), reflecting, as in many high-mol% G+C organisms, a strong bias toward a G or C residue in the first codon position for Arg and Leu and in the third position for all amino acids. This base composition makes DNA sequencing somewhat more difficult than for lower-mol% G+C organisms but renders the identification of the reading frame relatively easy.

Extrachromosomal elements. Like most bacteria, several *Frankia* strains have been shown to harbor plasmids (56, 80, 189, 191, 240, 242). Estimates of the proportion of strains carrying plasmids of various sizes have ranged from 10% or less (191, 240) to 62% in a screen of 16 isolates from *Myrica pensylvanica* (56). No functions have been ascribed to any of the plasmids, except in two instances in which *nif* hybridizable DNA was found associated with plasmid bands (82, 239). Some of the smaller plasmids are candidates to form the basis of cloning vectors, but, in the absence of a selection technique, little success has been achieved in introducing plasmids into other *Frankia* strains.

Genes Involved in Nitrogen Metabolism

Since traditional approaches are not yet available for studying *Frankia* genetics, most work has proceeded through the cloning of genes via heterologous hybridization to genes from other organisms, most notably those involved in nitrogen metabolism. These genes include the cloning and sequencing of *nifH* (184, 190), *nifD* (187, 271), part of *nifK* (271), *nifB*, *nifX*, *nifW*, and *nifZ*, open reading frames that correspond to the *Azotobacter vinelandii* orf3 and *Azorhizobium caulinodans* orf1 (13) regions (103), *glnA* (111), and *glnII* (209).

***nif* gene organization.** At least 20 *nif* genes are involved in N_2 fixation in the well-characterized *Klebsiella pneumoniae*, and many of these genes have homologs in other diazotrophs (74). The structural genes for the Fe protein, and the MoFe protein of nitrogenase are encoded by the *nifH* and the *nifD* and *nifK* genes, respectively. Hybridization results have indicated that *nifHDK* in some *Frankia* strains are clustered on the chromosome (170), as they are in most N_2-fixing bacteria (74). *F. alni* HFPArI3 and the closely related (if not identical) strain HFPCpI1 have been studied with regard to *nif* gene organization. In HFPArI3, *nifHD* and *nifK* are located in a region that contains sequences that hybridize to other *nif* genes, including *nifA* and *nifB* (241). The *nif* region of *F. alni* HFPCpI1 exhibits a restriction map and gene organization identical to that described for ArI3 (103). In HFPCpI1, five genes about 4 kbp downstream from *nifHDK* have been sequenced, four of which belong to a single operon consisting of at least orf3 orf1 *nifW*, and *nifZ*; *nifB* is located immediately downstream from *nifZ* and may be transcribed as part of another operon (103). NifB, NifW, and NifZ are all involved in FeMo-cofactor biosynthesis (74). The open reading frames orf3 and orf1 have been identified by similarity to open reading frames with unknown functions in *A. vinelandii* and *A. caulinodans*, respectively.

TABLE 2. Comparison of Shine-Dalgarno regions of *Frankia* genes

Strain	Gene	Sequence[a]	Reference
HFPCpI1	*glnA*	GACGCTGGAGGTCGA	111
HFPCpI1	*glnII*	CACGGGGAGTATTGA	209
HFPArI3	*nifH*	–AGGAGGAGCACCGC	190
HRN18a	*nifH*	CAGGAGGAGAAAGCA	184
HRN18a	*nifD*	GATGAGGTCCCGATC	184
HFPArI3	*nifD*	GATGAGGTCCCGACC	187
HFPCpI1	*nifB*	GAAGGACGGTCGACG	103
HFPCpI1	*nifW*	AAGGAGACCGTCGCG	103
HFPCpI1	*nifZ*	GACGGAGGAGGCCCG	103
HFPCpI1	orf1	GCGAGGTCGCCCGCG	103
HFPCpI1	orf3	GAAGGGAGACGCGGC	103

[a] Shown is the region from −15 to −1 before the inferred translational start site. The regions having sequence complementarity to the 3′ end of the *Frankia* strain ORS020606 (184) 16S rRNA are underlined.

All of the *nif* genes characterized thus far have been identified by sequence similarity to *nif* genes from other bacteria, and their coding regions have been circumscribed by the finding of ribosomal binding sites (Table 2), ATG or GTG start codons, and stop codons. Because of a lack of techniques for genetic manipulation, the functionality of all *Frankia nif* genes is not certain. However, multiple copies of *Frankia nif* genes have not been detected by hybridization methods, except in a single case where strain ARgP5^{AG} was found to have *nif*-hybridizable DNA on a large plasmid as well as on the chromosome. Thus, the chances are high that functional *nif* genes have been identified. With the availability of cosmids containing most of the *nif* genes, it is now possible to develop a map of the *nif* region of HFPCpI1 (HFPArI3) comparable to what has been constructed for other organisms. The characteristics of *Frankia nif* genes that make them worthwhile to pursue are that *Frankia* is the only high-mol% G+C gram-positive genus whose *nif* sequences are currently available for phylogenetic studies of gene organization, and their expression is intimately tied to the developmental cycle of *Frankia* vesicles.

***glnA* and *glnII*.** Genes encoding two GSs (*glnA* and *glnII*) have recently been cloned and sequenced from *F. alni* HFPCpI1 (111, 208, 209). Unlike other organisms that have two GSs and that have been studied, in this case the two GS genes are linked and are separated by 449 bp (111). Primer extension analysis of the *glnA* transcript revealed the presence of a single promoter, designated *glnAp*1 (111). Transcripts originated at two adjacent nucleotides, which is a common occurrence for the related *Streptomyces* species (252). A comparison of the *Frankia glnA* promoter region

with the *glnA* promoter from *S. coelicolor* revealed two areas of sequence similarity that corresponded to the −10,−35 region of both sequences. In addition, a series of direct repeats were clustered around the putative promoter, as is common in *Streptomyces* promoter regions. Thus, it appears likely that a promoter for a constitutive gene in *Frankia* strains has been identified. Limited similarity was found between the *glnA* promoter and *E. coli* or *Streptomyces-E. coli*-like promoters (119), which explains why the cloned *Frankia glnA* failed to complement an *E. coli glnA* deletion mutant until placed under the control of the *lac* promoter (111).

Primer extension analysis of the HFPCpI1 *glnII* gene, encoding GSII, revealed a promoter region, designated *glnII*p1, that has little sequence similarity to NtrA (σ^{54})-binding sites found in gram-negative bacteria (111). A comparison of sequences upstream of *glnII* from *Frankia* strains with the upstream sequence of *glnII* from *S. viridochromogenes*, for which a promoter has not yet been published, revealed one region of similarity that corresponded to the *glnII*pI of HFPCpI1 *glnII*, providing support for the identification of *glnII*p1 as a nitrogen-regulated promoter in *Frankia* strains. Interestingly, direct repeats were not found overlapping *glnII*p1, suggesting that the regulation of *glnII* differs in kind from that of *glnA*.

rRNA Gene Organization

Much of the work involving sequencing *Frankia* rRNA genes has been directed at clarifying phylogenetic relationships of *Frankia* strains with each other and with other bacteria (99, 178). A study of rRNA gene organization in the *Casuarina*-isolated *Frankia* strain ORS020606 revealed the presence of two copies the 16S-23S-5S rRNA gene cluster that had identical restriction maps (185). The rRNA genes did not differ markedly from previously described prokaryotic rRNA genes in their organization, but the work confirmed the phylogenetic position of *Frankia* strains established in previous studies. Two possible promoters were identified through their similarity with other rRNA promoters; the consensus derived from the promoters was TTGACA for the −35 region and TAAYYT for the −10 region. The mRNA attachment region had the sequence 5′-GGAUCACCUCCUUUCU-3′ (185). This sequence corresponds exactly to that found in *S. lividans* but differs in the last three positions from that of *E. coli* (227). Ribosome-binding sequences from the few genes that have been sequenced show typical Shine-Dalgarno sites that match the 3′ end of the 16S rRNA in four or more positions (Table 2) (229).

PROSPECTS

Studies on the microbiological aspects of *Frankia* species began in earnest when the isolation of the first strain was announced only 15 years ago. Much of the intervening time has been spent establishing techniques for growing *Frankia* strains and pursuing hypotheses concerning the nature of the organism, vesicles, spores, and physiology, developed from structural studies dating back to previous decades. Thus, a fair amount is now known about the phylogenetic position of *Frankia* strains, the structure and function of spores and vesicles, the physiology of carbon and nitrogen metabolism, ecology in the soil and nodule, and the structural features of the infection process; also, some details are beginning to emerge about the genetics of the microorganism.

Many opportunities for studies on the actinorhizal symbiosis are available. The research areas holding the most potential for future work include studies on the genetics and molecular genetics of host plant infection and symbiotic interactions, phylogenetic and taxonomic studies of *Frankia* strains from hosts that have yet to yield infective strains, and physiological studies directed at understanding the basis for symbiosis. Plant work is somewhat more difficult and time-consuming, but studies directed at the complementary physiology of the plant and identifying genes involved in nodule formation would help lay the foundation for a unifying hypothesis to explain why actinorhizal nodules form on certain, relatively distantly related plants but not on other plants within the same plant family. The identification of shared characteristics may provide insight into the coevolution of the two symbionts.

In a broader sense, the practical roles played by actinorhizal plants in many natural environments, especially in the context of global changes proposed to occur in the next few decades, are important to identify and perhaps exploit if necessary. Many actinorhizal plants grow in soils that have lost vegetation or nutrients, and they serve to ameliorate the effects of such losses. Thus, practical issues of the actinorhizal symbiosis must be addressed as part of an overall study of the biology of actinorhizal symbioses.

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